GLOBAL WATER PATHOGEN PROJECT

**PART THREE. SPECIFIC EXCRETED PATHOGENS: ENVIRONMENTAL AND
EPIDEMIOLOGY ASPECTS**

SALMONELLA, ENTERIC FEVERS, AND SALMONELLOSIS

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Summary

Salmonella species are widespread across the globe and associated with both human and animal hosts. The genus *Salmonella* includes the species *S. enterica*. *S. enterica* is further subdivided into subspecies amongst which *Salmonella enterica* subspecies enterica includes many of pathogens important for human disease. While newer molecular methods provide good species and subspecies level discrimination for *Salmonella*, many laboratories still rely on antigenic structure based on the initial Kauffman-White-Le Minor (KWL) scheme which remains valid.

In humans, *Salmonella* infections are most prevalent in low-to-middle income countries and are divided into infections caused by typhoidal and non-typhoidal salmonellae. Typhoidal salmonellae; *Salmonella enterica* serovars Typhi, Paratyphi A, B and C are restricted to human hosts and associated with invasive disease (enteric fever). Non-typhoidal salmonellae (NTS), which may be of zoonotic origin, are primarily associated with non-invasive disease. In both Africa and Asia, burden of salmonella infections is highest in children. *Salmonella* strains show geographical differences with different strain types being prevalent in different regions.

Clinically, enteric fever typically presents with fever and gastrointestinal symptoms. Complications include intestinal bleeding/perforation (in older children and adults) and paralytic ileus in younger children. Additional complications include cardiopulmonary disorders, bronchopneumonia, thrombocytopenia/anaemia (particularly amongst children in Africa), as well as sepsis. NTS infections are typically associated with gastroenteritis, although in immunocompromised (e.g., in patients with HIV), NTS may also result in invasive disease. It is suggested that genomic differences may be linked to non-invasive versus invasive behaviour of *Salmonella* strains.

The main reservoir for salmonellae is the gastrointestinal tract of vertebrates, with bacteria being shed in stool. Spread is through the fecal-oral route. Environmental factors; including contaminated food, water and poor hand hygiene, contribute to dissemination. Prevention of

Salmonella infections centers on safe water, sanitation and hygiene, food safety, and vaccination where available. Commercial vaccines are available for human use against the enteric or typhoid fever causing *S. Typhi*, and newer conjugate vaccines may have longer lasting immunity than capsular polysaccharide vaccine precursors.

Treatment technologies for reduction of *Salmonella* in wastewater fractions can be divided into three main types: chemical, biological and thermal. When comparing the inactivation of *Salmonella* spp. with *Escherichia coli*, the latter is somewhat more resistant to most treatments and can therefore be used as a proper indicator for salmonella during treatments. *Salmonella* has several genetically-driven responses to stress related to the inactivation treatments, which increase survival during extreme conditions. In this chapter the inactivation time for salmonella in relation to pH, ammonia concentration and temperature is presented. For pH, generated inactivation chemical substances aid in the inactivation: at higher pH uncharged ammonia is the most active molecule enhancing inactivation while at low pH carbonate and organic acids both increase the efficiency of inactivation. For heat inactivation, increased dry matter content increases the time of survival. Biological treatments affect the survival, while also decreasing the number of viable *Salmonella* over time. However, the effect of the biological treatment is difficult to monitor and quantify and therefore extended treatment durations are recommended for biological treatment if the treatment is not combined with chemical or thermal treatment.

1.0 Epidemiology of the Disease and Pathogen(s)

1.1 Global Burden of Disease

1.1.1 Global distribution

Salmonella infections with both typhoidal as well as non-typhoidal salmonella are prevalent across the globe. These infections are particularly associated with low-to-middle income countries in Africa (Table 1) and Asia (Table 2).

Table 1. Africa: *Salmonella enterica* serovar Typhi and invasive Non Typhoidal *Salmonella* (NTS) Burden

Region	Years	Adjusted Incidence/ 100,000 PYO (95%CI) ^a					Reference
Age Groups		0 to 1 yr	2 to 4yrs	5 to 14 yrs	<15 yrs	>15 yrs	Overall
S. Typhi							
Burkina Faso ^b (Nioko 11)	2012 to 2013	0 (0 to 0)	251 (107 to 590)	315 (191 to 519)	227 (148 to 350)	0 (0 to 0)	104 (68 to 161)
Burkina Faso ^b (Polesgo)	2012 to 2013	0 (0 to 0)	1,890 (1,202 to 2,972)	485 (263 to 896)	719 (500 to 1,035)	107 (46 to 252)	383 (274 to 535)
							Marks et al., 2017
							Marks et al., 2017

Region	Years	Adjusted Incidence/ 100,000 PYO (95%CI) ^a						Reference
Ghana (Asante Akim North) ^b	2011 to 2013	120 (49 to 290)	1,079 (762 to 1,528)	314 (230 to 430)	389 (310 to 486)	-	-	Marks et al., 2017
Guinea-Bissau (Bandim) ^b	2011 to 2013	0 (0 to 0)	53 (13 to 208)	18 (5 to 72)	20 (8 to 53)	4 (1 to 20)	10 (4 to 22)	Marks et al., 2017
Kenya (Kibera) ^b	2012 to 2013	148 (48 to 458)	490 (264 to 912)	489 (338 to 709)	419 (308 to 569)	141 (82 to 243)	284 (217 to 371)	Marks et al., 2017
Kenya (Kilifi) ^c	1998 to 2014	0.5% and 2.1% of bacteremia in children and adults respectively						Muthumbi et al., 2015
Madagascar ^b (Imerintsiatosika)	2011 to 2013	0 (0 to 0)	0 (0-0)	171 (181 to 360)	95 (45 to 201)	20 (4 to 103)	58 (29 to 114)	Marks et al., 2017
Madagascar ^b (Isotry)	2011 to 2013	0 (0 to 0)	0 (0 to 0)	62 (11 to 359)	42 (7 to 247)	42 (12 to 151)	42 (15 to 119)	Marks et al., 2017
Malawi (Blantyre)	1998 to 2014	S. Typhi associated with 1.2% of bacteremia; was most common in children and young adults, with a peak age range of 5 to 10 years. Number of <i>Salmonella</i> Typhi isolated from blood cultures peaked in 2013 with an incidence of 207 per 100,000 population.						Feasey et al., 2015
Nigeria (Kano)	2008 to 2015	A study of etiology of bacteremia in febrile children under 5 yrs: S. Typhi was isolated from 280/686 (40.8%) positive blood cultures. Total children screened (n = 1944).						Obaro et al., 2015
Nigeria (Federal Capital Territory)	2008 to 2015	A study of etiology of bacteremia in febrile children under 5 yrs: S. Typhi isolated from 84/457 (18.3%) positive blood cultures. Total children screened (n = 6082).						Obaro et al., 2015
Sudan (East Wad Medani) ^b	2012 to 2013	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	Marks et al., 2017
South Africa	2003 to 2015	Data collected as part of national surveillance showed that the annual incidence rates of typhoid fever subsequent to 2003 remained low; between 0.1 and 0.2 cases per 100,000 population						Keddy et al., 2018
Tanzania (Moshi) ^b Rural	2011 to 2013	0 (0 to 0)	0 (0 to 0)	18 (8 to 44)	18 (7 to 42)	28 (8 to 95)	20 (10 to 41)	Marks et al., 2017
Tanzania (Moshi) ^b Urban	2011 to 2013	0 (0 to 0)	1,028 (472 to 2,237)	103 (54 to 199)	155 (94 to 256)	201 (99 to 408)	168 (111 to 253)	Marks et al., 2017
Invasive Non typhoidal <i>Salmonella</i> (iNTS)								
Burkina Faso ^b (Nioko 11)	2012 to 2013	753 (460 to 1,233)	753 (460 to 1,233)	236 (133 to 420)	475 (352 to 640)	35 (13 to 96)	237 (178 to 316)	Marks et al., 2017
Burkina Faso ^b (Polesgo)	2012 to 2013	431 (162 to 1,147)	630 (288 to 1,380)	0 (0 to 0)	255 (138 to 470)	54 (16 to 179)	144 (83 to 249)	Marks et al., 2017
Ghana (Asante Akim North) ^b	2011 to 2013	1,733 (1,373 to 2,188)	1,908 (1,469 to 2,479)	147 (93 to 232)	742 (631 to 873)	-	-	Marks et al., 2017
Guinea-Bissau (Bandim) ^b	2011 to 2013	291 (176 to 482)	53 (13 to 208)	53 (14 to 97)	116 (69 to 161)	0 (0 to 0)	37 (24 to 57)	Marks et al., 2017
Kenya (Kibera) ^b	2012 to 2013	49 (7 to 350)	49 (7 to 348)	17 (2 to 124)	31 (10 to 95)	33 (10 to 101)	32 (14 to 70)	Marks et al., 2017
Kenya (Kilifi) ^c	1998 to 2014	32.6 (28.1 to 37.7)		2.4 (1.49 to 3.62)	1.7 (0.87 to 3.11)			Muthumbi et al., 2015

Region	Years	Adjusted Incidence/ 100,000 PYO (95%CI) ^a						Reference
Kenya (Kibera) ^b	2009 to 2014	In children 0 to 4 yrs: 735.2 (622.4 to 868.5) and overall 149.8 (128 to 175.2)						Verani et al., 2015
Kenya (Lwak) ^b	2009 to 2014	In children 0 to 4 yrs: 2305.6 (2,102.2 to 2,528.7) and overall 471.9 (436.7 to 510)						Verani et al., 2015
Madagascar ^b (Imerintsiatosika)	2011 to 2013	100 (18 to 562)	0 (0 to 0)	0 (0 to 0)	18 (3 to 99)	0 (0 to 0)	9 (2 to 50)	Marks et al., 2017
Madagascar ^b (Isotry)	2011 to 2013	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	Marks et al., 2017
Malawi (Blantyre)	1998 to 2014	iNTS serovars isolates from 6.1% of blood cultures, of which 79.1% were <i>S. Typhimurium</i> and 15.8% were <i>S. Enteritidis</i> . Frequency of iNTS peaked in <5 yrs and in 20 to 45 yrs age groups. During the study period isolations of <i>S. Enteritidis</i> peaked in 2001, and of <i>S. Typhimurium</i> in 2003.						Feasey et al., 2015
Nigeria (Kano)	2008 to 2015	A study of etiology of bacteremia in febrile children under 5 yrs. NTS isolated from 73/686 (10.6%) positive blood cultures. Total children screened (n = 1,944).						Obaro et al., 2015
Nigeria (Federal Capital Territory)	2008 to 2015	A study of etiology of bacteremia in febrile children under 5 yrs. NTS isolated from 26/457 (5.7%) positive blood cultures. Total children screened (n = 6,082).						Obaro et al., 2015
Sudan (East Wad Medani) ^b	2012 to 2013	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	Marks et al., 2017
South Africa (Gauteng Province)	2003 to 2013	Incidence of iNTS per 100,000 population/year dropped from 5.0 (2003) to 2.2 (2013)						Keddy et al., 2017
Tanzania (Moshi) ^b Rural	2011 to 2013	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	28 (8 to 95)	7 (2 to 23)	Marks et al., 2017
Tanzania (Moshi) ^b Urban	2011 to 2013	427 (125 to 1,461)	0 (0 to 0)	0 (0 to 0)	26 (8 to 88)	0 (0 to 0)	19 (5 to 64)	Marks et al., 2017

^aAdjusted Incidence/ 100,000 PYO (95% CI) included unless otherwise described; ^bThe incidence rate / 100,000 person year of observation (PYO) was adjusted for health-seeking behavior; ^cAdjustment based on missed blood cultures and contamination rate per age group.

Table 2. Asia: *Salmonella enterica* serovar Typhi, Paratyphi (A, B, C) Burden

Region	Year	Burden Incidence/100,000 Person/Years (Unless Otherwise Noted)	Reference
<i>S. Typhi</i>			
Bangladesh (Dhaka)	2003 to 2004	1050 (95%CI 680 to 1550) (<5 yrs) 90 (95%CI 50 to 140) (>5 yrs) 200 (95%CI 150 to 280) (Overall)	Naheed et al., 2010
Bangladesh (Dhaka)	2001 and 2014	0.9 to 3.9% and 3.3 to 8.8% Retrospective analysis of blood culture positivity rates from 3 hospitals	Saha et al., 2018

Region	Year	Burden Incidence/100,000 Person/Years (Unless Otherwise Noted)	Reference
China (Hechi)	2001 to 2002	15.3/100,000 (5 to 60 yrs) 29.3/100,000 (5 to 15 yrs) 12.4/100,000 (>16 yrs)	Ochiai et al., 2008
India	1950 to 2015	9.7% (95% CI 5.7 to 16%) prevalence rate of in hospital based studies, with Systematic and Meta-analysis 377 (95% CI 178 to 801)/100,000 patient year pooled incidence rate from community based studies from Delhi and Kolkata.	John et al., 2016
India (Kolkata)	2003 to 2004	214.2/100,000/year (overall) 89.2/100,000/year (0 to 1 yr), 340.1/100,000/year (2 to 4 yrs), 493.5 /100,000/year (5 to 15 yrs) 119.7/100,000/year (>16 yrs).	Ochiai et al., 2008
India (New Delhi) ^a	2010 to 2015	0.62% to 0.18%	Balaji et al., 2018
India (Vellor) ^a	2010 to 2015	1.38% to 0.17%	Balaji et al., 2018
India (Mumbai) ^a	2010 to 2015	0.4% (2010) and 0.2% (2015)	Balaji et al., 2018
Indonesia (North Jakarta)	2002 to 2003	81.7/100,000/year (overall) 0/100,000/year (0 to 1 yr), 148.7/100,000/year (2 to 4 yrs), 180.3/100,000/year (5 to 15 yrs) and 51.2/100,000/year (>16 yrs).	Ochiai et al., 2008
Pakistan (Karachi)	2002 to 2004	573.2/100,000 /year (2 to 5 yrs) 412.9/100,000 /year (5 to 15 yrs)	Ochiai et al., 2008
Pakistan (Karachi, Lahore, Islamabad)	1992 and 2015	6.42% and 1.32% Review of laboratory data from 3 tertiary care hospitals showed a decrease in S. Typhi positive blood culture rate from	Das et al., 2018
Nepal (Khatmandu)	2008 to 2012	2 to 8% blood culture positivity rate for S. Typhi. Retrospective analysis of patients presenting with fever to 11 hospital reporting.	Bajracharya et al., 2014
Thailand	2003 and 2014	8.6 cases/100,000 3.0 cases/100,000 Based on national surveillance data the incidence of typhoid fever reported to have decreased	Techasaensiri et al., 2018
Vietnam (Nation wide)	1999 to 2003	14.7/100,000 (>100/100,000 high incidence in 2 provinces (Soc Trang and Dien Bien) and (>10 <100/100,000 lower incidence in 18 other provinces).	Cuong, 2011
Vietnam (Hue)	2002 to 2004	21.3/100,000 (overall) 24.2/100,000 (5 to 15 yrs) 10.9/100,000 (>16 yrs).	Ochiai et al., 2008
Vietnam (Ho Chi Minh City)	1994 and 2015	12.3% and 0.07% blood culture positivity rate. in an infectious diseases hospital in Ho Chi Minh City, Review of published reports suggest a decreasing burden of S. Typhi.	Nga et al., 2012; Nga et al., 2018
S. Paratyphi A, B, C			
Bangladesh (Dhaka)	2003 to 2004	40 (95% CI 20 to 80)	Naheed et al., 2010
Bangladesh (Dhaka)	2001 and 2014	0.2 to 0.8% and 0.75 to 3% Retrospective analysis of blood culture positivity rates from 3 hospitals:	Saha et al., 2018
India	1950 to 2015	105 (95% CI 74 to 148)/ 100,000 patient year pooled incidence rate of from community based studies from Kolkata. 0.9% (95% CI 0.5 to 1.7%) prevalence rate Systematic review and Meta-analysis in hospital based studies	John et al., 2016
Pakistan (Karachi, Lahore, Islamabad)	1992 and 2015	1.29% and 0.39% Review of laboratory data from 3 large medical centers showed that blood culture positivity for <i>Salmonella enterica</i> Para A and B decreased	Das et al., 2018

Region	Year	Burden Incidence/100,000 Person/Years (Unless Otherwise Noted)	Reference
Nepal (Khatmandu)	2008 to 2012	2 to 3% blood cultures positive for <i>S. Paratyphi</i> Retrospective analysis of data in patients presenting with fever to 11 hospital reporting.	Bajracharya et al., 2014
Thailand	2007 and 2009	0.41 and 0.77 cases /100,000 persons/year Based on national surveillance data the incidence of paratyphoid fever remained stable during the study period	Techasaensiri et al., 2018

^aLaboratory data in 3 large medical centers reviewed to investigate proportion of *S. Typhi* positive blood culture.

In high income settings such as Western Pacific Region (Australia, Brunei, Japan, New Zealand and Singapore) *Salmonella* infections are primarily associated with gastrointestinal rather than invasive disease. Estimates from 2010 suggested that salmonellae contributed to 1% of waterborne gastrointestinal infections (Gibney et al., 2017). In the USA, 1872 cases of *S. Typhi* and 467 cases of *S. Paratyphi* were reported between 2008 and 2012 (Date et al., 2016), although the majority were travel associated. Notably *Salmonella* strains show geographical differences. The Non-typhoidal salmonellae (NTS) associated with invasive disease in Southern Africa are reported to be *S. Typhimurium* and *S. Enteritidis* with *Salmonella Typhimurium* pathotype ST313 primarily seen to be associated with invasive disease and febrile illness (Kariuki and Onsare, 2015). On the other hand, in the Lao People's Democratic Republic, studies of isolates collected between 2000 and 2012 showed the most frequent isolates associated with both non-invasive and invasive non-typhoidal salmonella (iNTS) disease included *S. Enteritidis*, *S. Typhimurium* as well as *S. Choleraesuis* (Le Thi Phuong, 2017).

The strains can evolve and change over time within countries and regions. Studies conducted in Nepal suggested that, in 2005-2006, several distinct genotypes of *S. Typhi* were present. More recent findings, however, show that this heterogeneity of genotypes, particularly in children under five, is now reduced. Such change is linked to ongoing clonal expansion of the *S. Typhi* H58 lineage. Further analyses reveal a shift in dominance from H58 Lineage I to H58 Lineage II, with the latter being significantly more common after 2010. Such change is likely due to spread from other South Asian Countries (Britto et al., 2018).

NTS are mainly associated with diarrhoeal infection. The first global estimates of the disease burden 1990-2010 reported that of all foodborne diseases, diarrheal and invasive infections due to non-typhoidal *S. enterica* infections resulted in the highest burden, causing 4.07million (95% uncertainty interval (UI) 2.49-6.27 million) Disability-Adjusted Life Years (Kirk et al., 2015).

A more recent Global burden of diseases study 2015 (Collaborators, 2017) reinforces that finding and estimates

that, globally, Rota virus, *Shigella* and *Salmonella* were among the top three causes of diarrhoeal deaths, with *Salmonella* infection contributing 90,300 deaths (95% UI 34,100-183,100).

1.1.2 Symptomology

Clinical features of enteric infections, including their complications and outcomes, differ between adults and children. Even among children, differences exist between infants and older children as well as between children in Africa and Asia (Azmatullah et al., 2015). The typical presentation is fever 5-15 days after exposure. Fever may be accompanied by gastrointestinal features and these can vary from diarrhoea, which is more common in infants, to constipation. Abdominal pain and nausea are more frequent in adults. Complications include gastrointestinal bleeds and perforations, which are related to the robust immune response and hence more common in older children and adults, while younger children are more likely to develop a paralytic ileus. Additional complications include seizures, cardiopulmonary disorders (myocarditis, endocarditis, pericarditis, and pericardial effusion) as well as bronchopneumonia. Transient pancytopenia in acute stage related to seeding of *S. Typhi* to the bone marrow is also recognized (Mai et al., 2003; Britto et al., 2017). Children in Africa have a higher risk of developing anaemia and thrombocytopenia relative to children from Asia (Azmatullah et al., 2015). Typhoid may also be associated with sepsis. A multinational study on causes of sepsis in Southeast Asia reported that 0.2% of sepsis cases were related to *S. Typhi* (Southeast Asia Infectious Disease Clinical Research Network, 2017).

While NTS are classically associated with gastrointestinal infections, in immunocompromised patients there is increasing recognition of their role in invasive disease. However, genomic differences in strains may also account for the difference in invasive versus enterocolitic manifestations, and influence host immune response, and these differences are subjects of active research (Gal-Mor et al., 2014).

Between 2003 and 2013, 278 cases of meningitis due to NTS (of which 44.5% (n=106) were due to *Salmonella Typhimurium*) were detected through a laboratory-based surveillance in South Africa and were shown to be

significantly associated with HIV disease (Keddy et al., 2015). Similar association of iNTS with HIV and sickle cell disease has been reported from Kenya. Analysis of these 192 iNTS isolates obtained 2005-2013, 114 were shown to be Typhimurium and 78 Enteritidis. *Salmonella* Typhimurium pathotype ST313 was primarily seen to be associated with invasive disease and febrile illness (Kariuki and Onsare, 2015). Use of antiretroviral therapy has been associated with reduction in disease due to invasive NTS (Keddy et al., 2017). These reports are supported by a recent literature review, spanning 1966-2014, which reported NTS as being responsible for up to 39% of community-acquired blood stream infections in sub-Saharan Africa (Uche et al., 2017). In Southeast Asia, 21% of sepsis is associated with invasive NTS (Southeast Asia Infectious Disease Clinical Research Network, 2017).

1.2 Taxonomic Classification

The genus *Salmonella* is included in the family Enterobacteriaceae, order Enterobacteriales (Adeolu et al., 2016). The taxonomy of salmonellae has evolved over the last quarter of a century towards a modern classification based on DNA-DNA homology (Popoff et al., 2004). Currently the genus *Salmonella* has two species: *Salmonella enterica* and *Salmonella bongori*. Six *S. enterica* subspecies are also recognized; *Salmonella enterica* subspecies: enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV), indica (VI) (Alikhan et al., 2018; Brenner et al., 2000; Johnson, 2018). *Salmonella enterica* subspecies are further sub-classified as 'serovars' based on the antigenic structure and reactivity. Subspecies I of *Salmonella enterica*, also known as *Salmonella enterica* subspecies enterica, contains many of the medically important human pathogens; however, rare infections with other subspecies have been reported.

The first compilation of classification based on antigenic structure, the Kauffman-White-Le Minor (KWL), scheme remains widely used as the gold standard for strain typing. This classification is based on the antigenic structure of salmonellae. The bacterium has somatic "O" and flagellar "H" antigens with significant variation in antigenic composition that allows identification in the laboratory based on antigen-based agglutination tests. The O antigen is the polysaccharide constituent of the lipopolysaccharide (LPS) of the bacterial cell wall; where a single bacterium may express more than one antigenic type of O on its cell surface (Wattiau et al., 2011). By contrast, only one "H" flagellar antigen is expressed on the bacterial cell's surface at any one time. However, the *Salmonella* genome may encode for 2 (mostly) or up to 3-4 flagellar antigens, where cultures in the laboratory can exhibit a switching of flagellar antigens called phase variation. Serovars determined by antigenic serotyping (antibody agglutination) in the laboratory are named in the KWL scheme, which is maintained by the WHO collaborating Center for Reference and Research on *Salmonella*. Updates for addition of new emerging variants to the latest classification scheme of 2007 were proposed in a research paper in 2014 (Issenhuth-Jeanjean et al., 2014).

The diversity, and phase variation of antigens in *Salmonella* have made antigenic classification difficult. Molecular typing based on genes encoding for O and H antigens has been performed (Wattiau et al., 2011). Further, many molecular methods with improved discrimination have also been proposed to replace serotyping (Alikhan et al., 2018). The genome structures of several medically important salmonellae are now accessible in GenBank, which could inform more specific molecular-based typing methods. However, due to labour and cost demands, KWL scheme-based serotyping remains in use. Many clinical laboratories shorten this serotyping scheme to perform a few agglutination reactions to identify the most common serotypes. Application of rapid methods including Matrix Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) Mass Spectrometry, and molecular methods such as Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), and Multiple-Locus Variable-Number Tandem Repeat Analysis (MVLA) also enable typing, but these methods have varying accuracy and discriminatory ability. Rare and emerging serotypes are still only identifiable in reference laboratories through either exhaustive antigenic serotyping or molecular serotyping. Such methods are necessary to establish new pathogenic serotypes, detect and confirm outbreaks, and delineate epidemiology.

1.2.1 Pathogenic salmonellae

Salmonellae have a widely-distributed host range including both human and animal species. Two distinct disease processes are known in humans: invasive and non-invasive. Non-invasive infections manifest most commonly as gastroenteritis, whereas invasive infections comprise bacteremia - including the syndrome of 'enteric fever' - and meningitis. Enteric fever pathogens, also known as typhoidal salmonellae, are restricted to human hosts and have no animal reservoir (Eng et al., 2015). Other zoonotic salmonellae occasionally cause invasive infections in immunocompromised hosts. Typhoidal salmonellae include: *Salmonella enterica* serovars Typhi, Paratyphi A, Paratyphi B, and Paratyphi C. Reasons for the predilection of typhoidal salmonellae to cause invasive bacteremic infections remain putative; however, the consensus leans toward their avoidance of the human natural immunity by suppression of neutrophilic killing as one of the major escape mechanisms leading to invasiveness (Hiyoshi et al., 2018).

Non-typhoidal salmonellae (NTS), while occasionally responsible for invasive infections in those with weak immunity, mostly induce gastrointestinal inflammation leading to gastroenteritis. Although many new serovars are increasingly recognized, more commonly identified ones are *Salmonella enterica* subspecies enterica serovar Enteritidis (*Salmonella* Enteritidis), and *Salmonella enterica* subspecies enterica serovar Typhimurium (*Salmonella* Typhimurium). Several emerging strains causing zoonotic and foodborne salmonellosis among humans have been described. Among the more common ones observed in the 2000-2007 WHO analysis of data from quality assured laboratories are: Infantis, Agona,

Heidelberg, Virchow, Newport, Hadar, Montevideo, and Saintpaul (Hendriksen et al., 2011).

1.3 Transmission

Transmission routes and reservoirs are important control points for salmonellosis and therefore an understanding of these epidemiological aspects is essential to devise control measures to prevent illness.

Salmonellae enter their hosts through the gastrointestinal tract and multiply therein to either cause inflammation directly (resulting in gastroenteritis) or invade the intestinal epithelium to establish systemic illness (Pegues and Miller, 2010). The route of transmission is therefore fecal-oral, with reservoirs propagating the cycle through fecal shedding and hosts becoming infected through oral intake. The primary reservoirs for salmonellae are gastrointestinal tracts of vertebrates, with environmental contamination occurring through shedding of bacteria in feces (Kingsley and Baumler, 2000). Transmission is facilitated and sustained through environmental reservoirs such as contaminated food, water, and soil, although direct transmission through contaminated hands (with feces from human or animal hosts) also occurs. This environmental transmission is enabled by the prototrophic nature of salmonellae; they require little or no nutrients to thrive in environmental or food reservoirs (Rhodes and Kator, 1988).

One important feature of *Salmonella* transmission cycle is asymptomatic fecal shedding by vertebrate hosts. This occurs in both animals and humans, usually following an infection, and continuing in the recovery phase (Gopinath et al., 2012). This shedding may continue in the long term, giving rise to 'salmonella carriers' or chronic excretors in the population that serve as important reservoirs of infection. Based on murine models of *S. Typhimurium*, it has been proposed that transmission is reinforced due to 'super-shedders' in the population, excreting larger amounts of the organisms in their feces due to persistent inflammation and high abundance of organisms in the intestinal lumen (Monack, 2012).

Asymptomatic NTS carriage rate of 2.0-2.9% in the general populations, with 4.4% reported amongst chicken farmers in Vietnam (Trung et al., 2017). While the exact pathogenesis of persistence is unclear, a long term follow-up of NTS cases in Israel showing an overall persistence rate of 2.2% suggests that 65% of the persistent cases had experienced prolonged symptoms and relapsing diarrhoea (Marzel et al., 2016).

Since NTS have a wider host range, transmission is efficiently maintained through multiple direct and indirect routes where zoonotic reservoirs are present. NTS gastroenteritis is recognized as predominantly a food-borne illness, although water remains an environmental source leading to food contamination (Hohmann, 2001). Where the zoonotic reservoirs are food animals, transmission occurs through contaminated food as vehicles: poultry, eggs, pork, fish, shellfish, beef, milk, and dairy products (Carrasco et al., 2012). Where domestic or companion animals are in

contact with fresh produce, fresh fruit and vegetables, and juices contaminated with animal feces are efficient vehicles of transmission. Where such raw material is used in preparation of packaged foods, retail food products that become contaminated also act as sources of transmission and can lead to significant outbreaks due to centralized large-scale production (Tauxe, 1991). In all such instances, poor sanitation practices, poor population hygiene, and poor manufacturing and packaging practices instigate the transmission cycle. Use of water contaminated with NTS for irrigation in horticulture and to wash fresh produce also leads to propagation (Islam et al., 2004), as a variety of salmonellae have been recovered from surface water and groundwater sources (Levantesi et al., 2012).

Recent evidence also suggests that invasive vs diarrheal strains of salmonellae are distinct genotypically and phenotypically, and differentially adapted to environmental reservoirs (MacKenzie et al., 2019). Mutations altering biofilm formation in strains affect adaptability to environmental reservoirs, and strains causing invasive disease have been found to 'switch off' biofilm genes. The triggers and possible therapeutic and engineering control implications of this phenotypic difference between invasive and gastroenteritis-causing strains require further study.

As highlighted earlier, typhoidal salmonellae are exclusively adapted to human hosts and therefore transmission cycles are also restricted to humans. In contrast to NTS, typhoidal salmonellae are not considered primarily foodborne, but also understood to spread through drinking water. Presence of *S. Typhi* in drinking water, surface water and groundwater has been demonstrated as well as linked with outbreaks (Gauld et al., 2019; Qamar et al., 2018). Potable water contaminated with human feces or sewage therefore serves as a critical link in the transmission chain with potential to serve as a hazard control point (Dewettinck et al., 2001).

The incubation period of most salmonellosis is 12 to 72 hours, but may be as long as one week (Humphrey, 2000). However, when the infective dose is high, incubation periods of as short as 2.5 hours have been observed. Both NTS and typhoidal salmonellae have been subjected to volunteer studies to establish infective doses. Earlier studies carried out three quarters of a century ago demonstrated infective doses as high as 100,000 cells of NTS to establish infection in volunteers with later studies demonstrating an even higher required dose. Such doses are unlikely to occur in food or water, and practically smaller doses can establish infection as bacteria are protected by food particles. Typically, infective doses associated with dairy, chocolate, and processed meat are lower due to inherent capacity to inactivate and overcome the host stomach acid barrier. Individuals with impaired gastric acidity are therefore at higher risk of contracting illness (Humphrey, 2000).

The presence of salmonellae in aquatic environments due to contamination with human and animal excreta is an important instigator of the natural transmission cycle. Precautions taken at the consumer level or population level

to minimize food contamination are therefore necessary to prevent human infections.

1.4 Population and Individual Control Measures

In addition to the high burden of typhoid fever in many parts of the world, growing antimicrobial resistance in *Salmonella* Typhi is a major concern (Bhutta et al., 2018a; 2018b). Recent reports of XDR Typhi strains that are resistant to first line agents (Cotrimoxazole, Ampicillin, Chloramphenicol) as well as to fluoroquinolones and to third generation cephalosporins has raised alarm bells. In settings where these strains prevail, treatment options are limited to Azithromycin and carbapenems (Klemm et al., 2018). Azithromycin resistance has also been reported

(Ahsan and Rahman, 2018) and the possibility of XDR Typhi acquiring additional resistance to Azithromycin is a feasible concern.

In view of increasing limited treatment options, efforts to control the spread of the disease within communities requires urgent attention. Major control strategies include; vaccination and hygiene efforts.

1.4.1 Vaccines

1.4.1.1 *S. Typhi* vaccines

Currently three vaccines are recommended for *S. Typhi* (WHO, 2018a, 2018b) (Table 3).

Table 3. Vaccines for *S. Typhi* (Adapted from WHO, 2018a)

Vaccine	Vaccine Composition	Administration	Efficacy	Duration of protection
Typhoid conjugate vaccine (TCV)	Vi polysaccharide antigen linked to tetanus toxoid protein (also referred to as Vi-TT conjugate vaccine)	Intramuscular; single dose (0.5 mL) in Adults and children >6 months to <45 years of age.	Vaccine "sero-efficacy": 85 to 87% [95% CI: 80 to 88] Voysey and Pollard, 2018; Jin et al., 2017	Evidence suggests protection may persist for up to 5 years after primary immunization.
Unconjugated Vi polysaccharide (ViPS)	Purified Vi capsular polysaccharide from the Ty2 <i>S. Typhi</i> strain.	Intramuscular/ subcutaneous, single dose. Adults and children >2 years of age	Effectiveness in children aged 5 to 16 years: 57% [95% CI: 6 to 81] Khan et al., 2012; Sur et al., 2009	Revaccination recommended every 3 years
Live attenuated Ty21a	A live attenuated Ty2 strain of <i>S. Typhi</i> in which several genes have been attenuated. The resulting vaccine strain, termed Ty21a, lacks the Vi antigen	Enteric coated capsules administered orally: 3 doses every second (alternate) day. (USA and Canada 4 doses). Adults and children >6 years of age.	Efficacy for 3 oral doses of Ty21a: 67 to 80% [95% CI: 16 to 95] Jin et al., 2017; Darton et al., 2016; Levine et al., 1999	Australia and Europe recommend re-vaccination every 3 years for people living in endemic areas, and yearly for travelers to these areas. While USA recommends vaccination after 5 years and Canada after 7 years in people at exposure risk.

Currently there are no approved vaccines for Paratyphoid. Protection against *S. Paratyphi* B following Ty21a vaccine was reported in a large scale trial in Santiago, Chile (Levine et al., 1999). This finding however has not been confirmed by other studies. Ty21a vaccination was further shown not to confer protection against *S. Paratyphi* A in a trial in Indonesia (Simanjuntak et al., 1991).

1.4.1.2 NTS vaccines

The increasing realization of the high burden of invasive

NTS disease and associated morbidity, particularly in Africa, has triggered efforts towards developing a vaccine for these infections (Tennant et al., 2016). These efforts have been encouraged by findings suggesting that antibodies against NTS protect against invasive NTS disease (Gordon, 2011). A number of vaccine candidates targeting *S. Typhimurium* and *S. Dublin* are in the process of development (Haselbeck et al., 2017). However, NTS vaccine efforts face challenges including paucity of NTS disease data from Asia and South America. The major challenge though is the large number of NTS serovars circulating, raising concerns that strains targeted by a

vaccine would be replaced by other circulating strains. In other words, there is concern that widespread use of a vaccine against *S. Typhimurium* and *S. Enteritidis* would result in serovar replacement, e.g., by *S. Dublin* (Haselbeck et al., 2017).

1.4.2 Hygiene measures

Intake of unsafe water and contaminated food has been implicated in several outbreaks of typhoid fever (Luby, 2018). Contamination of municipal water or borehole water with sewage or other contaminated water is well recognized to contribute to spread of typhoid in many settings (Baker et al., 2011). Given the role of contaminated water and food in transmission of *Salmonella*, access to safe water, adequate sanitation as well as food safety including hygiene among food handlers are important pillars of typhoid control.

Improvements to water supplies, including filtration and chlorination, have been shown to reduce the burden of typhoid fever and lead to its elimination. In an outbreak in Tajikistan in 1997 associated with inadequate treatment of municipal water supplies, the re-introduction of chlorinated water supplies was effective in leading to a marked decline in typhoid fever cases (Mermin et al., 1999).

Similarly, the Metropolitan Region of Chile faced a large outbreak of typhoid fever from 1975-1983. In response, between 1983 and 1984 the country instituted a number of measures to prevent the spread of enteric fever. These included typhoid vaccination, treatment of chronic *S. Typhi* carriers, and educational campaigns about hygienic handling of crops and food. In 1991, as part of efforts to control cholera, crop irrigation with sewage contaminated water was stopped, leading to improvement of water quality and irrigation practices. New irrigation channels were constructed to avoid sewage discharge and water channels were chlorinated. Human activity near water sources was prohibited and sanitary barriers were established (Marco et al., 2018). Impact analysis suggests that interruption of person to person transmission through the 1983-1984 measures reduced disease rates by 51%. However, introduction of environmental measures in 1991; through control of irrigation waters and crop cultivation

and interrupting the contact of sewage-contaminated items with the public led to a 77% decline in typhoid fever. This decline has continued so that today the typhoid fever is rare in the region (Marco et al., 2018).

Evidence therefore points to a need for a multipronged approach to controlling diseases associated with *Salmonella enterica* serovars Typhi, Paratyphi A, Paratyphi B, and Paratyphi C as well as with NTS serovars. Access to safe water and food, as well as improved sanitation and sewage disposal, are key. These measures need to be implemented along with a system aimed at early case detection with appropriate treatment and vaccination of at risk populations.

2.0 Environmental Occurrence and Persistence

2.1 Detection Methods

2.1.1 Culture-based methods

Salmonellae are gram-negative, flagellated, non-lactose fermenting, facultative anaerobic bacilli, of which most are motile. Most of the *Salmonella* serovars grow at a temperature range of 5-47°C, with the optimum being 35-37°C, and in the pH range 4-9, with the optimum range of pH 6.5-7.5. Salmonellae require high water activity for growth (Aw 0.94-0.99) (Graziani et al., 2017). Current testing of food and environmental samples for the presence of salmonellae can be divided into three stages: (i) detection of *Salmonella* spp.; (ii) identification of the species and its specific serovar designation; and (iii) subtyping of the isolate. For the detection and isolation of salmonellae, culture-based methods still dominate, with several media used in both food microbiology and for environmental sampling (Table 4). Most traditional culture-based methods for detection and isolation of salmonellae generally involve nonselective pre-enrichment, followed by a selective enrichment step, followed by plating onto selective agars, and finally biochemical and serological confirmation of presumptive colonies (Lee et al., 2015). Table 4 lists culture media and selective/differentiating compounds currently in use for food and environmental monitoring and indicates when media are recommended by standards/guidelines for environmental monitoring.

Table 4. Culture-based media used for *Salmonella* detection and identification in food and environmental samples. Letters indicate corresponding standards/guidelines that recommend the media for environmental sampling (mandatory or optional).

Enrichment media	
Non Selective Enrichment Media	Selective Enrichment Media
Buffered peptone water (BPW) ^{a,c,d}	Diagnostic semi-solid <i>Salmonella</i> agar (DiaSalm)
Lactose broth (LB)	Gram-negative Hajna broth
ONE Broth- <i>Salmonella</i>	Modified Semi-solid Rappaport-Vasiliadis (MSRV) medium ^{b,d,e}
Phosphate buffered saline (PBS) solution ^g	Muller-Kauffmann Tetrathionate Novobiocin (MKTTn) broth ^{c,d}
Salmosysts broth base	Manitol selenite broth
Tryptic soy broth (TSB) ^{b,e}	Peptone-Novobiocin broth ^a
Modified tryptone soya broth ^a	Rappaport-Vassiliadis (RV) medium ^a
Universal pre-enrichment (PU) broth ^f	Rappaport-Vassiliadis Soya Peptone (RVS) medium ^{c,d}
	Selenite Cystine (SC) broth ^{a,d,f}
	Simple method <i>Salmonella</i> (SMS) agar
	Tetrathionate (TT) broth ^a
Selective/differential Plate Media	
Traditional Plate Media	Chromogenic Plate Media
<i>Salmonella-Shigella</i> (SS) agar ^d	chromID® <i>Salmonella</i> / <i>Salmonella</i> Identification medium II (SMID II) ^d
Brilliant green (BG) agar ^{c,d}	SM-ID agar/SMID2/CHROMOID2/Rambach agar ^a
Brilliant green-phenol red-lactose-sucrose (BPLA) agar ^a	BBL CHROMagar
Bismuth-sulfite (BS) agar ^{c,d*,f}	<i>Salmonella</i> SMS -agar
Hektoen enteric (HE) agar ^d	RAPID <i>Salmonella</i> plate
MacConkey agar	SALSA plate
Miller-Mallinson (MM) ^{d,f}	ASAP <i>Salmonella</i> ^d (C8)
Xylose-lysine-deoxycholate (XLD) agar ^{a,b,c,d,e}	Harlequin <i>Salmonella</i> ABC/ ab-Chromogenic (ABC) medium ^d
Xylose Lysin Tergitol (XLT4) agar ^d	Oxoid Brilliance <i>Salmonella</i> Agar (Oxoid)/Oxoid <i>Salmonella</i> Chromogenic medium II ^d
Deoxycholate citrate lactose agar (DCLS) ^d	Chromogenic <i>Salmonella</i> esterase (CSE) ^d
Deoxycholate citrate agar DCA (Leifson agar) ^d	HiChrome™ <i>Salmonella</i> agar
Mannitol lysine crystal violet brilliant green (MLCB) agar ^d	RaiHans
Selective/inhibitory Reagents	Biochemical/differential Tests (<i>Salmonella</i> Reaction, Predominant*)
bile salts	O-nitrophenyl-b-D-galactopyranoside (ONPG) test (β -galactosidase negative) ^{c,d}
bismuth sulfite	H ₂ S production (*positive) ^f
brilliant green	Indole test (Tryptophanase negative) ^{a,d,f}
cefsulodin	L-lysine decarboxylase (LDC) medium (decarboxylase negative) ^{a,c,d}
cycloheximide	Lysine iron agar (LIA)(decarboxylase negative, *H ₂ S positive) ^{b,e}
sodium deoxycholate	Malonate utilization (negative)
ferric ammonium citrate III	Potassium cyanide (KCN) (growth inhibition)
malachite green, nitrofurantoin	Kligler Iron agar (KIA) (*Lactose-, sucrose +)
novobiocin, sulphacetamide	Triple sugar iron (TSI) agar (*Lactose -, Sucrose -, Glucose +, *H ₂ S positive) ^{b,c,d,e}
sulfadiazine	Urea hydrolysis (urease negative) ^{a,b,c,d,e,f}
sulphamethazine	Voges Proskauer test (Acetoin production negative) ^f
sulfapyridine	Analytical Profile Index (API) 20E (kit with 20 miniaturized biochemical tests) ^{a,c,f}
tetrathionate	C8-esterase
tergitol	
sodium thiosulphate	
ferrioxamine E	
Oxyrase™	
Inhibigen™	

^aCEN/TR 15215, 2006 Characterization of sludges - Detection and enumeration of *Salmonella* spp. in sludges, soils, soil improvers, growing media and biowastes - Parts 1-3; ^bUSEPA, 2006 Method 1682: *Salmonella* in Sewage Sludge (biosolids) by Modified Semisolid Rappaport-Vassiliadis (MSRV) Medium (EPA-821-R06-14); ^cISO 19250, 2010 Water quality -- Detection of *Salmonella* spp.; ^dISO 6579, 2017 Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella*, Parts 1-3; ^eUSEPA, 2012. Method 1200: Analytical Protocol for Non-Typhoidal *Salmonella* in Drinking Water and Surface Water (EPA. EPA 817-R-12-004); ^fUSEPA, 2010. Standard Analytical Protocol for *Salmonella* Typhi in Drinking Water (EPA/600/R-10/133)

Culture-based methods are still the most widely used for salmonellae testing of environmental samples due to their sensitivity and selectivity. A major drawback is that 5–7 days is typically required to obtain a result, especially because multiple culture- and identification steps are required. Chromogenic and fluorogenic growth media (Table 4) for detection, enumeration, and identification directly on the isolation plate will make the test result using these selective media typically available at least 1 day earlier than conventional methods (Mooijman, 2012). These media are in general as sensitive as conventional culture media but improved in regard to readability. Attempts to shorten the total culture time for the isolation of *Salmonella* spp. are ongoing, often by combining culture steps. For example, in Salmosysts broth and the ONE Broth-*Salmonella*, the pre-enrichment and the selective enrichment are combined in one culture step (Mooijman, 2012). When such enrichment is combined with a chromogenic medium (e.g., in Qxoid *Salmonella* PreciS; Simple Method *Salmonella* SMS or BioRad 7 methods) presumptive *Salmonella* can be recognized in 42 h to 3 days. There is also a rapid API kit available for enterobacteriaceae differentiation (Rapid 20 E), which performs the biochemical testing in 4 hours compared to the typical 18–24 h (BioMérieux).

Lee et al. (2015) and Silva et al. (2018) both reviewed methods for salmonellae detection and identification for the purpose of food safety and emergency response and provide a comprehensive list of commercially-available biochemical tests for salmonella confirmation. For culture media formulation and how to interpret responses to media and tests presented in Table 4, we refer to U.S. FDA Bacteriological Analytical Manual (BAM) Chapter 5: *Salmonella* (Andrews et al., 2011). Mooijman et al. (2012) also present a comprehensive description about culture media used to detect salmonellae in food microbiology. Further details are found in Appendix A

2.1.2 Molecular methods

Salmonella can also be detected via molecular methods targeting macromolecules (e.g., DNA, RNA, proteins) specific to the pathogen. Generally, molecular methods for salmonellae can be divided into immuno-based assays and nucleic acid based-assays, although some methods, such as lateral flow assays and biosensors, are applied to a wide range of compounds (Bahadir and Sezginürk, 2016). An

enrichment step is often used to concentrate salmonellae prior to rapid molecular confirmation, e.g., using immuno-based assays, which can shorten the time for analysis. Depending on the enrichment method, analysis time can be reduced by as much as 48 hours. Nucleic acid based-assays, on the other hand, are designed to serve as a means of primary detection and screening, rather than for viability confirmation. They can be used to help determine if viable but non-culturable (VBNC) cells exist but cannot be used to directly determine viability or virulence. Details are found in Appendix A

2.1.3 Applicability for environmental sampling

Detecting and enumerating salmonellae in environmental samples is particularly challenging, especially when seeking to differentiate strains and serovars. Most works to date apply culture with PCR or other confirmation, but there are some reports of direct analysis of environmental samples using PCR-based methods. Environmental samples are typically complex and variable matrixes are prone to interfere with analysis media. Additionally, the number of salmonellae cells in the samples may be low, whereas other environmental bacteria may be abundant. Addressing the diversity of bacteria with selective media may in turn not allow injured cells to grow. Similarly, inhibitors, such as humic acids and metals, that are co-extracted with DNA or RNA can interfere with subsequent PCR-based analyses. There are reference methods for analysis of salmonellae in a wide range of environmental samples, including: sewage sludge (USEPA, 2006; SIS-CEN 15216, 2006); wastewater (ISO, 2010), drinking water (ISO, 2010; USEPA, 2012; USEPA, 2010), surface waters (ISO, 2010; USEPA, 2012; USEPA, 2010) and soil (SIS-CEN 15216, 2006). For soil, sediments, and other environmental matrices that may not be directly covered by a specific standard, the U.S. EPA/FDA Selected Analytical Methods for Environmental Remediation and Recovery (SAM) (Campisano et al., 2017) do suggest methods for sample preparation and subsequent analysis by culture or PCR (Table 5). The latest ISO standard method for salmonella in the food chain, ISO 6579 (2017) is applicable for samples from the primary production stage such as animal feces, dust, and swabs (ISO 6579 - Part 1) and also guide on the procedure for serotyping *Salmonella* serovars, independent of the source from which they are isolated (ISO 6579 - Part 3) (Table 5).

Table 5. *Salmonella* spp. concentrations in manure samples

Area	Matrices	Salmonella Serovar or Gene	Percent Positives (# of Samples)	Concentrations CFU or GC/g ^a Average (Min to Max)	Reference
Australia	Cattle, fresh	invA	32% (6)	2.5E+03 GC	Klein et al., 2010
Ireland	Pig, infected only	S. Typhimurium	(20)	NR ^b (1.0E+00 to 1.0E+04)	Lynch et al., 2017

Area	Matrices	Salmonella Serovar or Gene	Percent Positives (# of Samples)	Concentrations CFU or GC/g ^a Average (Min to Max)	Reference
UK	Cattle, fresh	<i>Salmonella</i> spp.	7.7% (810)	3.9 E+04 (NR to 5.8E+05)	Hutchison et al., 2004
UK	Pig, fresh	<i>Salmonella</i> spp.	7.9% (126)	9.6E+03 (NR to 7.8E+04)	Hutchison et al., 2004
UK	Poultry litter, fresh	<i>Salmonella</i> spp.	17.9% (67)	5.0 E+03 (NR to 2.2 E+04)	Hutchison et al., 2004
UK	Sheep, fresh	<i>Salmonella</i> spp.	8.3% (24)	1.1E+03 (NR to 2.0E+03)	Hutchison et al., 2004
USA	Poultry litter, stored 4 weeks	<i>Salmonella</i> spp.	NR (6)	NR (2.0E+02 to 1.4E+05)	Hruby et al., 2018

^aConcentrations as CFU (Colony Forming Units) unless noted as GC (Gene Copies); ^bNR: Not Reported

Sewage fractions, due to their nature or treatment (e.g. alkaline stabilization), potentially have low or high pH and can contain considerable amounts of ammonia, which may be toxic to the salmonella cultivation if the pH in the culture media is/will become alkaline during incubation. The effect on culture media from the matrix will be most crucial during the pre-enrichment step. The U.S. EPA (2006) guidelines for sewage sludge analyses suggest initial adjustment of sludge pH to 7.0-7.5. Different pre-enrichment media (LB, BPW, UP, M-9, TT and RV) applied to environmental poultry samples was found to result in different pH, with LB having the lowest pH of 4.7, whereas in UP, M-9, and BPW, the lowest pH was 6.1-6.4 (Cox et al., 2018). U.S. EPA reference methods for drinking and surface waters suggest dilution with double strength pre-enrichment solution when pre-enriching water samples to account for dilution effects. Filtration can also be used to overcome influences from complex sample matrixes, which could interfere with the media and is by ISO 15215:1 (2006) recommended for resuscitation of sub-lethally stressed bacteria for sludge samples with TS up to 20%. The standard/technical report by the European committee for standardization (CEN) for characterization of sludges, soils, soil amendments, growth media and biowastes recommend that a novobiocin-supplemented peptone water is used as a primary medium to suppress competing microflora. Similarly, to the ISO19250 (2010) water standard, direct inoculation in selective media is recommended when waste water is analyzed. Among non-selective enrichment media in the standards/methods listed in Table 5 are buffered peptone water (BPW) and Tryptic soy broth (TSB) for primary enrichment of salmonellae in general and universal pre-enrichment broth (UP) for *S. Typhi* (Table 4).

For the nucleic acid based methods, DNA can be extracted for downstream PCR and qPCR to determine the presence/absence and quantity of the target gene. Method development for the type of environmental sample of interest is required to ensure that an appropriate amount of sample is collected. Significant dilution may also be required to reduce the effects of PCR inhibitors. Messenger-RNA (mRNA) using reverse transcription PCR (RT-PCR) can be used to better assess the viable population due to short half-life of mRNA and detection of mRNA from housekeeping genes are indicative for active transcription. For culture-based methods, elevated nutrients and temperature (sometimes suggested stepwise 10°C at a time) are applied to rescuing VBNC state and generally stressed cells. Baloda et al. (2001) did find the conventional culture method (pre-culture in enriched, buffered peptone water followed by the use of selective media) to be more reliable and sensitive method for complex environmental samples compared to molecular methods such as *Salmonella*-specific PCR. For plate culturing, the cells can be subjected to less stress by overlaying selective agar with a nonselective agar, e.g. overlaying XLD with TSA. Standard methods are still being established in nucleic methods and genes used for detection in environmental samples vary (Table 5).

Karkey et al. has shown success in extracting from utilizing the Metagenomic DNA Isolation Kit for Water and diluting extracted DNA 1:20 (Karkey et al., 2016). This, in conjunction with enrichment plating successfully identified *Salmonella* in well waters.

2.2 Occurrence in the Environment

2.2.1 Animal manure

Agricultural livestock are widely recognized as carriers of *Salmonella* spp. (Jacobsen and Bech, 2012) and salmonellae have been isolated from numerous species of farm animals, including poultry, cattle, pigs, and sheep (Abulreesh, 2012). It is predominantly serovars of *S. enterica* subspecies enterica that infect warm blooded animals (including humans). The non-enterica subspecies of *S. enterica* are considered to be related to cold-blooded animals and human infections more common in

immunocompromised individuals (Lamas et al., 2018). Salmonellae appearing as commensals in farm animals, but not associated with infection in humans in the same region, indicates that not all salmonellae are of clinical interest (Lamas et al., 2018). Whereas some salmonellae infect mainly one or few species, some are highly zoonotic and infect humans as well as animal hosts. For some serovars, the animals act as asymptomatic carriers whereas the salmonella causes illness in human. Table 6 includes, for comparison with excreta/sewage fractions, some references on concentrations of salmonellae in animal manure.

Table 6. *Salmonella* spp. in urine, black water, latrine and septic tank sludge

Area	Matrices	Salmonella Serovar or Gene	Percent Positives (# of Samples)	Concentrations ^a CFU or MPN/ ml or g Average (Min to Max)	Reference
Australia	Urine, human, individual samples	Non typhoidal <i>Salmonella</i>	NR ^b (23)	>1.0 E+05 /ml	Paterson et al., 1997
Malawi	Latrine sludge, Fossa alternas	<i>Salmonella</i> spp.	NR (11)	3.46E+02 (<1 to 1.2 E+03) /g	Kumwenda et al., 2017
Malawi	Latrine sludge, Urine dry diverting toilets	<i>Salmonella</i> spp.	NR (13)	5.09E+02 (<1 to 3.2E+03) / g	Kumwenda et al., 2017
South Africa	Latrine sludge, Ecosan toilets	<i>Salmonella</i> spp.	NR (NR)	2.2E+05 (NR) /g dry matter	Jimenez et al., 2006
Sweden	Black water, 100 pec	Stanley; Bareilly	NR (NR)	NR (0.2E+01 to 0.35E+01) /mL	Nordin and Vinneras, 2015
USA	Urine, human, individual samples, all infected	Montevideo; Oranienberg; Senftenberg; Meleagridis	NR (65)	NR (>1.0E+05-1.0E+04) /mL	Abbot et al., 1999
Vietnam	Septic tank sludge	<i>Salmonella</i> spp.	60% (20)	5.79E+02 (9.0E+01 to 1.9E+03) MPN /g dry matter	Yen-phi et al., 2010

^aConcentrations as CFU (Colony Forming Units) unless noted as MPN (Most Probable Number); ^bNR: Not Reported; ^cPE: Person Equivalent

2.2.2 Human excreta

Most salmonellae infecting humans are excreted via the faeces, but *S. Typhi* and *Paratyphi* are, during the phase of typhoid and paratyphoid fevers, excreted in the urine

(Schönning and Stenström, 2004). However, NTS cases of urinary tract infection has been recorded with concentrations of >100,000 colony forming units mL⁻¹ urine (Allerberger et al., 1992; Abbott et al., 1999). Even if salmonellosis is among the common causes of enteritis in

humans, studies report mainly prevalence and duration and not faecal excretion rates. There are a few publications reporting presence of salmonellae in source-separating sanitation systems. Concentrations of culturable salmonella in a black water system in Sweden (Nordin and Vinneras,

2015) was rather low compared to latrine sludge samples in South Africa (Jimenez et al., 2007; Jimenez et al., 2006) and Malawi (Kumwenda et al., 2017), despite the fact that in the latter studies samples were taken after a 12 months waiting period (Table 7).

Table 7. *Salmonella* spp. in raw and treated sewage

Area	Matrices, Level of Treatment	<i>Salmonella</i> Serovar or Gene	Percent Positives (# of Samples)	Concentrations CFU or MPN / 100ml ^a Average (Min to Max)	Reference
Raw Wastewater					
Poland	1 facility ^b , 315,000 PE ^c , sampled over 1 yr	<i>S. Typhimurium</i>	100% (12)	NR (2.5E+05 to 1.43E+06)	Kacprzak et al., 2015
Poland	1 facility ^b , 315,000 PE, sampled over 1 yr	bipA	100% (12)	(1.1E+06 to 5.81E+07 GC)	Kacprzak et al., 2015
Spain	1 facility, sampled over 7 months	<i>Salmonella</i> spp.	NR (21)	2.67E+02 MPN (NR)	Howard et al., 2004
Taiwan	9 hospital facilities ^b	<i>Salmonella</i> spp.	33.3% (27)	NR (1.0E+02 to 1.0E+05)	Tsai et al., 1998
Zimbabwe	1 facility, 100,000 PE	<i>Salmonella</i> spp.	65% (NR)	1.0E+08 (NR)	Gopo and Chingobe, 1995
Treated Wastewater Effluent					
Poland	1 facility, 315,000 PE, activated sludge sampled over 1 year	<i>S. Typhimurium</i>	50% (12)	NR (2.0E+04 to 1.47E+05)	Kacprzak et al., 2015
Poland	1 facility, 315,000 PE, activated sludge sampled over 1 year	bipA	75% (12)	NR (0 to 2.8+06 GC)	Kacprzak et al., 2015
South Africa	2 facilities, activated sludge process, chlorination, sampled over 1 year	<i>Salmonella</i> spp.	NR (>24)	NR (1.0E+03 to 2.0E+05)	Odadjare and Olaniran, 2015
Spain	1 facility, activated sludge process, sampled over 7 months.	<i>Salmonella</i> spp.	NR (21)	4.5E+01 MPN (NR)	Howard et al., 2004
Zimbabwe	1 facility, modified activated sludge process	<i>Salmonella</i> spp.	52% (NR)	<1E+02	Gopo and Chingobe, 1995

^aConcentrations as CFU (Colony Forming Units), unless noted as MPN (Most Probable Number); ^bWastewater treatment facility; ^cPE: Person Equivalent

2.2.3 Sewage/ wastewater and wastewater effluent

Systems collecting mixed waste water are generally larger than source-separating systems and the material is likely to contain salmonellae. In Sweden, it has been suggested that with more than 5,000 people connected to a sewage system, *Salmonella* can be expected in the waste water (Albihn and Stenstrom, 1998). However, even though the reported human salmonella prevalence /incidence in Sweden is rather low, salmonella has been detected in

black water from rather small systems (<100 PE) (Nordin and Vinneras, 2015; Nordin et al., 2018).

When *Salmonella* concentrations have been studied at different points in wastewater treatment systems, in general a decrease in concentration is observed as treatment progresses (Howard et al., 2004). However, the reduction is typically not sufficient to eliminate salmonellae, which are frequently found in treated effluent, even after disinfecting treatment including chlorination and

UV-treatment (Kinde et al., 1997; Santiago et al., 2018). Due to variation over locations, the concentration in treated effluent is for some studies higher than in the incoming wastewater in other locations/studies.

2.2.4 Biosolids/Sludge

Of the sewage fractions /products sewage sludge are most thoroughly analyzed with respect to salmonella. *Salmonella* spp. are frequently found in sewage sludge where concentrations as high as 10^7 cfu per ml has been reported for raw sludge (Parmar et al., 2001; Sahlstrom et al., 2004)(Table 8). Post storage of sewage sludge re-population may occur and has been related to changes in water content, e.g. rain fall (Zaleski et al., 2005).

Table 8. *Salmonella* spp. in sewage sludge

Area	Matrices, Sludge Treatment	Percent Positives (# of Samples)	Concentrations CFU or MPN/ g or ml ^a Average (Min to Max)	Reference
Brazil	5 facilities, sampled over 1 year, MAD followed by dewatering and liming (3 of 5 facilities)	39% (54) ^b	NR (0.43 to 1.22E+01) / g dry matter	Krzyzanowski et al., 2014
Canada	1 facility, MAD	NR (NR)	1.5E+07 CFU/ ml (NR)	Parmar et al., 2001
France	1 facility, raw sludge	100% (3)	NR (1.2 to 3.2)/ g dry matter	Pourcher et al., 2005
Mexico	1 facility, 35 L/s, raw sludge, sampled over 8 months	100% (32) ^d	NR (9.5E+06 to 1.4E+08) / g dry matter	Jiminez et al., 2002
Taiwan	9 hospital facilities, various sludge treatments	37% (27)	NR (<1 to 9.2E+04) CFU/g total solids	Tsai et al., 1998
USA	2 facilities, MAD or aerobic digestion followed by drying for 23 to 30 weeks	NR (NR)	NR (1.0E+01 to 1.0E+02) CFU/ g (growth to >1.0E+05 after rain)	Zaleski et al., 2005
USA	1 facility, raw sludge	100% (14)	1.14E+03 (1.0E+02 to 7E+04) / g	Gerba et al., 2008
USA	1 facility, MAD	100% (14)	1.05E+02 (1.0E+00 to 4.8E+02) / g	Gerba et al., 2008
USA	4 facilities, MAD, 3 sampling campaigns over 10 months	33% (6) ^e	NR (1.2E-01 to 2.4E-01 /g)	Wong et al., 2010
USA	1 facility, raw sludge	100% (1)	NR 2.44E+02 / g	Wong et al., 2010
USA	6 facilities, raw sludge from settling	100% (NR)	6.3E+02 (2.17E+02 to 1.0E+03) g dry matter	Dahab and Surampalli, 2002
USA	3 facilities, raw sludge from activated sludge process	100% (NR)	6.0E+02 (4.0E+02 to 7.5E+02) / g dry matter	Dahab and Surampalli, 2002
USA	6 facilities, MAD	100% (NR)	(1.00E+00 to 5.20E+01) / g dry matter	Dahab and Surampalli, 2002

^aConcentration given as MPN (Most Probable Number) unless noted as CFU (Colony Forming Unit); ^bWastewater treatment facility; ^cSerotypes identified: Infantis; Agona; Anatum; Corvalis ; Mbandaka; Typhimurium ; München; Salamae; Give; Javiana ; Minnesota; Molade; Newport; Ohio; Oranienburg; Ouakan; ^dGC: Gene Copy; ^eSerotype identified S. Typhi; ^fSerotype identified S. Enterica

2.2.5 Waters

Salmonellosis in general is considered to be a foodborne disease, mainly infecting through fresh produce, although the actual source of the salmonellae may be contaminated water. Water contaminated with human feces is one of the main transmission routes. Even if most serovars are considered to be foodborne, *S. Typhi* and *S. Paratyphi* are considered to be waterborne. Salmonellae has been detected in different countries and in a diverse range of

water sources, ranging from pristine (e.g. Patchanee et al., 2010; Till et al., 2008)) and low impacted water (e.g. Patchanee et al., 2010; Jokinen et al., 2010; Meinersmann et al., 2008) to heavily impacted water sources (e.g. Jyoti et al., 2010) (Table 8). *Salmonella* contamination occurring in surface water used for recreational purposes (Till et al., 2008); as a source of drinking water (Till et al., 2008); and for irrigation (Gannon et al., 2004). Increasing evidence indicates that irrigation water is a key source (or a vehicle) for transmission of *Salmonella* (Liu et al., 2018).

Table 9. *Salmonella* spp. in environmental samples, soil, and water fractions

Area	Matricies	Percent Positives (# of Samples)	Concentrations CFU or GC/ 100mL ^a	Reference
Australia	Rain Water	(6)	1 to 3.8E+01 GC ^a (invA gene)	Ahmed et al., 2010
Bangladesh	Soil	NR ^b	1.0E+02 to 5.0E+02	Alam and Mohammad, 2012
India	River water	87% (8)	1.0E+04 to 1.0E+06	Jyoti et al., 2011
Indiac	River Water	(18)	2.32E+02 to 5.89E+02 GC (staG gene)	Rani et al., 2014
Indiac	River Water Sediments	(18)	2.8 to 6.6 GC/g- (staG gene)	Rani et al., 2014
Saudi Arabia	Soil recreational and agricultural areas	15.5% (220)	NR	Abdel-Monem and Dowidar, 1990
Saudi Arabia	Poultry manure	NR	37 /g	Hruby et al., 2018
South Africa	River water	Upstream sewage plant, sewage contaminated	1.0E+02 to 3.5E+02	Odjadjare and Olaniran, 2015
USA	Lake and river water	42% (19)	0.2 to 1.7 MPN ^d	Byappanahalli et al., 2009
USA	Pond water in a water shed	NR	0.01 to 0.34 MPN	Jenkins et al., 2008

^aCFU (Colony Forming Units) and GC (Gene Copies) per 100 ml unless otherwise noted; ^bNR: Not Reported; ^c*S. Typhi* *Salmonella* serovar; ^dMPN: Most Probable Number

Salmonellae frequently occur in sewage-impacted fresh and marine water environments and their detection in the water environment have been correlated with proximity to the sewage discharge area (Jacobsen and Bech, 2012; Alonso et al., 1992; Baudart et al., 2000). They have also been linked to the intensity of rainfall, suggesting importance of transport of salmonellae by overland flow and animal rearing (Phan et al., 2003). However, other studies have found as many salmonellae positive samples upstream as

downstream of sewage effluent discharge (Gopo and Chingobe, 1995) and sometimes even higher concentrations upstream (Odjadjare and Olaniran, 2015). Studies have shown that salmonellae are able to colonize the non-host environment by forming stable, dividing populations and that a stable aquatic presence not solely be the consequence of continuous bulk transfer from human and animal sources.

2.2.6 Soil

Salmonella has been detected frequently in soils from both agricultural and recreational areas (Abdel-Monem and Dowidar, 1990; Thomason et al., 1975a; Thomason et al., 1975b)(Table 8). *Salmonella* survives for long periods and has also been found colonizing soil related to environmental and ecological niches as plant tissue and soil flagellates (Xuan Thanh et al., 2012). The presence of salmonellae in agricultural soils has been linked to use of irrigation water and organic fertilizers, such as sewage sludge and manure. It has also been shown that salmonellae can come from other sources besides irrigation or manure-derived fertilisers, such as wild birds. The same has been found in recreational land and in school yards where the salmonellae occurrence has been linked to the presence of wild animals (Phan et al., 2003; Haddock and Nocon, 1993; Haddock and Nocon, 1986).

2.3 Persistence

Compared to other bacteria, *Salmonella* spp. have high survival rates in aquatic environments and have been shown to have better survival strategies than *E. coli* in the natural environment (Winfield and Groisman, 2003).

The long persistence of *Salmonella* clones on farm premises (Baloda et al., 2001; Sandvang et al., 2000; Twiddy et al., 1988) indicates that the treatment of manures as well as other measures to prevent environmental contamination are necessary.

There is evidence to indicate that the survival of *Salmonella* spp. in organic-containing substrates, e.g. faecal sludge and manure, is lower than that in buffer solution. For example, Olsen and Larsen (1987) found 10-25% lower survival of *Salmonella* spp. in manure than for the same bacteria in the treatment capsuled in nylon bags added where the bacteria were kept in tryptic soy broth. However, Smith et al. (2005) observed the opposite, i.e. higher survival of *Salmonella* spp. in TSB than in sewage sludge at 55°C, although some chemical substances, not investigated, could have affected the survival in the sludge.

The survival of *Salmonella* spp. in soil is determined by various factors, including temperature, moisture, soil type, presence of plants, exposure to sun (UV) light, protozoan predation (Garcia et al., 2010), and competing microbial community members (Schierstaedt et al., 2016; Abd-Elall and Maysa, 2015). Salmonellae have been reported to survive for more than one year (Davies and Wray, 1996), with longer survival in clay soil than sandy soils (Nicholson et al., 2005) and in soils amended with organic material (Ellis et al., 2018; Paluszak et al., 2003). Salmonellae survival in soil has been correlated to soil moisture and temperature (Garcia et al., 2010; Pepper et al., 1993; Danyluk et al., 2008). However, Ongeng et al. (2015) reviewed survival of *Salmonella* in manure-amended soils and inconsistencies among survival times in soil, likely reflecting environmental

variability of differences in methodology. Further, they noted that persistence under field conditions are inconsistent compared to controlled environmental studies, where often one factor is singled out. It was also highlighted that, for many survival studies, populations potentially removed from soil by leaching are often not accounted for (Ongeng et al., 2015). Many studies from the field do not quantify the concentrations of salmonellae in soil, but merely detect its continued detection. Persistence of *Escherichia coli* and *Salmonella* spp. in surface soil following application of liquid hog manure for production of pickling cucumbers has also been documented (Cote and Quessy, 2005). *Salmonella* was also still present in one plot at the beginning of harvest time in the last year of the experiment, with a maximal persistence of 54 days in loamy sand and 27 days in sandy loam.

3.0 Reductions by Sanitation Measures

3.1 Biological Treatment

3.1.1 Latrines, septic tanks and wastewater treatment

Removal may be similar to *E.coli*, please see Pathogenic members of *Escherichia coli* & *Shigella* spp. Shigellosis for further information.

3.1.2 Excreta, biosolids and manure

3.1.2.1 Anaerobic digestion

Thermophilic anaerobic digestion at temperatures of 53°C and above brings about a rapid reduction in *Salmonella* spp., with D-values below one hour in all cases (Figure 1). During mesophilic (30-45°C) treatment, the reduction is very inconsistent and ranges from no reduction during 20 days of treatment (Smith et al., 2005; Shih, 1987), to a D-value of 1.7 days (Olsen and Larsen, 1987). Olsen and Larsen (1987) also found a great difference in treatment efficiency between 30 and 35°C. At colder temperatures (<30°C), rapid inactivation is reported in some studies (Cote et al., 2006), while others report limited to no reduction during the treatment (Figure 1). Compared with *Salmonella* spp., *E. coli* cells are more sensitive to the environment and are thereby inactivated faster during anaerobic digestion than cells of *Salmonella* spp. (Figure 1).

Figure 1. Decimal reduction (D) values (days) for 90% inactivation of *Salmonella* spp. (S) and *Escherichia coli* (E) during anaerobic digestion at temperatures from 24 to 55°C in batch, semi-continuous, semi-continuous or other reactors (average values from different treatment methods) (Abd-Elall and Maysa, 2015; Davies and Wray, 1996; Loewen and Hengge-Aronis, 1994; Spector and Kenyon, 2012; Lianou and Koutsoumanis, 2013; Pin et al., 2012; Erickson et al., 2014; Vinneras, 2013; Scaglia et al., 2014; Sidhu et al., 2001; Elving et al., 2014; Shih, 1987; Cote, Masse and Quessy, 2006; Termorshuizen et al., 2003).

Increased retention time within treatment increases the reduction effect. For example, Yen-Phi et al., (2009) showed that increasing the retention time from 3 to 30 days increased the reduction in *S. Senftenberg* 775W by 1 log₁₀. However, in mixed systems it is important to have a minimal retention time within the process, i.e. the shortest time from a material entering the reactor until it exits or, in fully mixed fed batch reactor systems, the time between loadings. To ensure pathogen inactivation within an anaerobic system at mesophilic or psychrophilic temperature, the treatment needs to be validated regarding the effect upon salmonellae, while thermophilic treatment requires time and temperature validation to confirm salmonella inactivation.

3.1.2.2 Fly larvae/vermicomposting

Fly larvae composting is rapidly gaining popularity as an alternative treatment method within faecal sludge management (Lalander et al., 2019; Lalander et al., 2013; Mutsakatira et al., 2018). The treatment is mainly performed using larvae of black soldier fly (*Hermetia illucens*) but can also be performed using larvae of other fly species, such as common housefly (*Musca domestica*) or

blowfly (family Calliphoridae).

All studies show a decrease in salmonellae numbers during fly larvae treatment compared with untreated controls. In *S. enteritidis*, Erickson et al. (2004) found a 1 log₁₀ reduction within 3 days and an even greater reduction with longer treatment time. In *S. Typhimurium* in faecal matter, Lalander et al. (2013) found a reduction of over 6 log₁₀ in 8 days in treatment with larvae, compared with just over 1 log₁₀ reduction during storage at room temperature. Those authors also found that 8 days of treatment were enough to ensure that no detectable salmonellae remained in any of the material, including inside and outside the larvae, from a starting concentration above 6 log₁₀ per g of faeces (Lalander et al., 2013).

Investigations into the mechanisms responsible for *Salmonella* spp. inactivation during fly larvae treatment indicate that the larvae treatment process generate anti-bacterial substances, either from the larvae or from bacteria in the process, decreasing the number of bacteria inside the larvae gut (Mumcuoglu et al., 2001), as well as protein or lipid substances that have an additional effect in treating the substrate. No studies have been performed on the risk of re-growth in the material during post-treatment

storage. However, the indication of excretion of antibacterial substances by the larvae indicates continued kill-off of any salmonella present.

Vermicomposting has similar effects on *Salmonella* spp. to fly larvae composting (Lalander et al., 2013; Lalander et al., 2015). The main difference is the actual vermicomposting process, which is more often run as a continuous process, with the risk of cross-contamination from migrating worms. In summary, fly larvae composting in batches during at least one week is sufficient to reduce *Salmonella* spp. to very low concentrations. The compost material and the larvae will then be safe from a *Salmonella* contamination perspective. Batch-wise vermicomposting has similar effects, but takes a longer time for full removal of *Salmonella* spp.

3.1.2.3 Composting

The main effect within composting is related to heat inactivation. The composting itself may have some effect in reducing salmonella numbers during the process, due to a combination of heat and chemicals produced during degradation, such as ammonia, carbonate and organic acids (Singh et al., 2012). The self-heating of the compost is very effective in sanitizing the material, but to achieve this effect it is important to keep the heat within the compost. This is best done by having a large enough pile/windrow (at least 1.2 m high) or an insulated reactor (Haug, 1993). However, there may be major differences in temperature in different parts of the compost, so to have an efficient removal of salmonellae, the compost needs to be turned several times so that all the material is treated (Berry et al., 2013). As a rule of thumb, reactors should be mixed three times and windrows should be mixed five times with time between turnings long enough for pathogen inactivation within the thermophilic area (Vinneras, 2013; Vinneras et al., 2010).

The main effect of the composting process derives from stabilisation of the material and from providing competing bacteria to decrease the risk of re-growth of salmonella that have survived the process or that have been carried in by vectors or by cross-contamination within the process. Elving et al. (2010) compared the risk of re-growth of *S. Typhimurium* during composting of food waste and manure in relation to the level of maturity of the compost, measured with Solvita™ tests. They found an inverse correlation between compost maturity and rate of *Salmonella* re-growth. On comparing different temperatures, they found that at mesophilic temperatures (37°C), *Salmonella* growth in less mature compost was fast, increasing by 2-3 log₁₀ during the first days of incubation and thereafter decreasing slowly, but never to below the initial count during the 8 days of the study. *Salmonella* growth was slower at psychrophilic temperatures (14°C), taking up to 8 days to reach the same level as at 37°C. In matured composts, there was no growth or in some cases even a decrease in *Salmonella* concentration (Elving et al., 2010). Miller et al. (2013) observed similar effects, with growth of 1 log₁₀ during the first day at 24°C in material with a lower stabilization level (bone meal) than manure-based fertilizers, which are normally composted for a very long time prior to use.

In summary, composting is a good stabilisation method and can prevent re-growth and re-contamination by *Salmonella* spp. (Sidhu et al., 2001; Elving et al., 2010; Miller et al., 2013). For sanitation, temperatures above 50°C are required, combined with mixing, as the material can have an uneven temperature distribution. Repeated mixing during the thermophilic phase ensures that most of the material reaches the higher temperature required to inactivate microbes. To reach a high level of pathogen removal, most of the material needs to be treated, e.g. a 5 log₁₀ reduction requires 99.999% of the material to be treated at a high enough temperature and that no re-growth occurs in zones with lower temperatures.

3.2 Disinfection

In treatment of substrates to reduce the *Salmonella* spp. content, when using natural substrates like faecal sludge and different sewage fractions it is difficult to isolate the single pathogen inactivation factor. Inactivation can be due to a combination of temperature, pH and toxic chemical substances, e.g. ammonia or organic acids. The survival of *Salmonella* spp. cells is regulated by several different stress responses that protect the cells in the environment, but all these are energy consuming and thus the survival will also depend on the availability of energy to the cells. For most treatments evaluated, *Escherichia coli* can be used as a somewhat more sensitive indicator of treatment efficiency regarding inactivation of *Salmonella* spp.

3.2.1 pH (lime, ammonia)

The two main chemical treatment systems used in water and wastewater disinfection are high pH and oxidation. Liming is a classic high pH treatment that has been used on sludge, surfaces and liquids for over a century. The goal of liming is to raise the pH of the matrix above 10, a value at which most organisms cannot survive. In high pH treatments, the effect of increased pH can be separated into two different functions, one related to ammonia (NH₃) concentration and one related to pH. As higher pH allows for a larger proportion of ammonia to be present in the uncharged toxic form, ammonia has a faster effect than just elevated pH alone, as it affects microbial cells that prefer lower pH. However, if only a small pH elevation is achieved by liming, the treatment can still be effective, because a small increase in pH leads to higher concentrations of basic, biocidal matrix constituents, such as ammonia and carbonate. These substances in turn contribute to pathogen inactivation. Lime treatment thus has multiple routes by which inactivation can be achieved, although they are all ultimately based on raising the pH.

Mechanisms of inactivation: High pH inactivates *Salmonella* cells by raising the intracellular pH, which ultimately leads to collapse of intracellular functions. Alternatively, the organisms may starve because all of their energy must be spent on actions to compensate for the high extracellular pH. For example, most non-extremophile bacteria maintain a stable internal pH of about 7.4-7.9, but survive or even grow over a considerably larger external pH range of 5.5-9 (Padan et al., 2005). At higher pH levels, cation/proton antiporters in most non-extremophile bacterial cell membrane export cations such as sodium (Na⁺), in exchange for importing hydrogen (H⁺). This energy-

consuming process is employed to maintain the intracellular pH within an optimal range. In combination with other stress factors, such as low redox potential and high temperatures, the sensitivity to high extracellular pH increases. Therefore, treatment with burnt lime (CaO) can be considerably more efficient than treatment with slaked lime (Ca(OH)₂), as the burnt lime increases not only the pH, but also the matrix temperature.

Ammonia has a rapid effect on *Salmonella* cells even at low concentrations (Figure 2). Inactivation of *Escherichia coli* by ammonia is similar to inactivation of *Salmonella* spp. and therefore *E. coli* can be used as an indicator for inactivation of *Salmonella*. An ammonia concentration of 10mM is enough for inactivation, with decimal reduction values (D-values) lower than 100 days and in most cases considerably lower than 10 days. The advantage with ammonia treatment is that the ammonia is not consumed during the process and, as long as ammonia remains in the matrix, in combination with elevated pH, there is no risk of re-growth or re-contamination.

Figure 2. Decimal reduction (D) values (minutes) for 99% inactivation of *Salmonella* spp. and enterohaemorrhagic *Escherichia coli* O157 (EHEC) in relation to the concentration of uncharged ammonia (NH₃) (Ellis et al., 2018; Paluszak et al., 2003; Pepper et al., 1993; Arrus et al., 2006; Semenov et al., 2009; Danyluk et al., 2008; Ongeng et al., 2015; Cote and Quessy, 2005; Olsen and Larsen, 1987; Smith et al., 2005; Padan et al., 2005; Vinneras et al., 2008; Vinneras et al., 2003; Ottoson et al., 2008).

3.2.2 Acids

There are two levels of acid-related inactivation of salmonellae: low pH and use of organic acids with higher chemical toxicity than inorganic acids. Organic acids are often used in food preservation, with good success (Huss et al., 2017), while direct use of organic acids for treatment of wastes is less common. A number of studies show that presence of organic acids assists in microbial inactivation

during other biological treatments, such as anaerobic digestion. As acids in the environment play an important role in inactivation of salmonella, several defence mechanisms have developed in the bacteria. According to Loewen and Hengge-Aronis (1994), there are similarities in gene expression of the response of *Salmonella* spp. regarding thermal and chemical stress, where the sigma factor independently, or together with other regulatory transcription factors, is responsible for the chemotactic response. The stress responses of the cells result in a

completely different structure and activity within cells, which enter a form of dormant stage during stress (Spector and Kenyon, 2012). Spector and Kenyon (2012) show that seven different stress responses are involved in managing acid stress, while only four are involved in managing heat stress. The genetic σ HSE factor is involved in three of these responses, for management of both acid and heat stress. However, in a study examining the inactivation kinetics of 60 *S. enterica* strains, Lianou and Koutsoumanis (2013) found no correlation between *Salmonella* spp. survival in relation to heat or acid stress (pH 3 (HCl), 37°C) or in relation to aryl hydrocarbon receptor (AMR) and the acid resistance of a specific strain. Lianou and Koutsoumanis (2013) also evaluated the acid sensitivity of 30 *Salmonella* strains at pH 3 during 4 hours at 37°C and found that growing the strains in an acid environment decreased the reduction rate in most cases, while 23 out of 30 strains also showed acid resistance build-up in *S. enterica*, indicating that it is strain-dependent. The inactivation rate at pH 3 at 37°C varied between a decimal reduction in 42 minutes and up to 4.8 hours (Lianou and Koutsoumanis, 2013). In another study, a moderate decrease in pH (to pH 5) was not sufficient to create a hysteresis reaction in the cells of *S. Typhimurium* and no specific gene activity was detected after neutralising the medium to pH 7 (Pin et al., 2012). Erickson et al. (2014) developed a model for *Salmonella* inactivation at mesophilic composting temperatures related to pH and time with temperatures above 40°C, where the main effect of the inactivation was related to production of volatile fatty acids. The study showed a decimal reduction in 7 to 37 hours (the lower the pH, the

faster the inactivation of *Salmonella*), indicating that the uncharged acid is the toxic form. As with the other chemical treatments, higher temperature increased the chemical-induced inactivation rate, mainly due to more permeable membranes (Erickson et al., 2014).

The effect of acids needs to be generally quantified in future studies. When using acids for *Salmonella* inactivation, each treatment system needs to be validated regarding the actual inactivation rate in that system. Important factors to be monitored in this regard are pH, temperature, and type of acid.

3.2.3 Thermal treatment

The effect of heat treatment on *Salmonella* spp. varies widely depending on the material treated. Many studies have examined heat inactivation of the microbes, but most consider heat treatment of food and feed-related substrates, rather than wastes. Most report the D-value or z-value (number of degrees by which the temperature has to be increased to achieve a 1 log₁₀ reduction in D-value), irrespective of the substrate. The lowest temperature at which inactivation has been detected is 47.5°C (Elving et al., 2014) and some studies report effective inactivation at 50°C, but most previous studies have been performed at temperatures of 55°C and above (Figure 3). In addition, some data on *E. coli* have been produced in studies examining whether it can be used as a model for *Salmonella* regarding heat inactivation. The data show that *E. coli* can be used as model for heat inactivation of *Salmonella* spp., as the inactivation rates (D-values) are within the same range for both bacteria types. However, *E. coli* seems to correspond to the lower range of D-values reported for *Salmonella* spp. (Buzrul and Alpas, 2007; Jin et al., 2008; Lang and Smith, 2008; Rakowski, 2012)(Figure 3).

Figure 3. Decimal reduction (D) values (minutes) for 90% inactivation of *Salmonella* spp. as a function of treatment temperature during thermal inactivation for substrates with different total solids (TS in percent) content (Elving et al., 2014; Buzrul and Alpas, 2007; Jin et al., 2008; Lang and Smith, 2008; Rakowski, 2012; Soldierer and Strauch, 1991; Eckner, 1992; Duffy et al., 1995; OrtaRamirez et al., 1997; Veeramuthu et al., 1998; Murphy et al., 1999; Mazzotta, 2000; Murphy et al., 2000; Smith et al., 2001; Bolton et al., 2003; Kumar and Kumar, 2003; Manas et al., 2003; Murphy et al., 2003; Spinks et al., 2006; Juneja, 2007; Osaili et al., 2007; Ma et al., 2009; Monfort et al., 2011; Kim et al., 2012; Burns et al., 2016; Hildebrandt et al., 2016).

Duffy et al. (1995) found a major difference in the survival rate of *S. Typhimurium* heat-treated in a buffer solution at a particular temperature in relation to the number of other competing organisms, with higher survival with higher concentrations of other bacteria. In that study, the initial concentration of *Salmonella* was 6 log₁₀ and the number of competing organisms increased from zero to 8 log₁₀ during the heat treatment. Velliou et al. (2013) observed similar effects when using higher counts of *S. Typhimurium*, with a population of about 8 log₁₀ being more heat tolerant, i.e. having a higher D₅₅-value, than less dense solutions. *S. Senftenberg* 775W has been used in several studies and in some cases shows higher thermal resistance than other strains tested

(Jarvis et al., 2016). However, when looking at all evaluated *Salmonella* spp. compared with *S. Senftenberg*, there is no trend for higher D-value in the latter (Figure 4). Other factors during treatment have a greater impact on the survival at different heat treatment temperatures. One factor found to have a substantial impact on the survival of *Salmonella* spp. is the stage of bacterial growth at the start of the trial, where bacteria at the lag phase have a higher survival rate than bacteria at the log stage. Other important factors are the number of bacteria in the material (where a higher bacteria count (8 log₁₀) either of *Salmonella* spp. or other bacteria, results in increased survival), the composition of the substrate itself, and how the heat is transferred within the material (Figure 4).

Figure 4. Decimal reduction (D) values (minutes) for 90% inactivation of different *Salmonella* strains in material with a dry matter content <30% heat-treated at different temperatures. Cocktail of all strains, with and without *S. Senftenberg*, is compared (Elving et al., 2014; Buzrul and Alpas, 2007; Jin et al., 2008; Lang and Smith, 2008; Rakowski, 2012; Soldierer and Strauch, 1991; Eckner, 1992; Duffy et al., 1995; OrtaRamirez et al., 1997; Veeramuthu et al., 1998; Murphy et al., 1999; Mazzotta, 2000; Murphy et al., 2000; Smith et al., 2001; Bolton et al., 2003; Kumar and Kumar, 2003; Manas et al., 2003; Murphy et al., 2003; Spinks et al., 2006; Juneja, 2007; Osaili et al., 2007; Ma et al., 2009; Monofort et al., 2011; Kim et al., 2012; Burns et al., 2016; Hildebrandt et al., 2016).

Duffy et al. (1995) concluded that the longer survival in some *Salmonella* species is related to the cells being in a stationary phase mediated by the genetic σ factor, thereby increasing the thermal resistance of the organism. Within species, the heat resistance of *S. Senftenberg* is greater when it is stressed by heat shock after overnight heat adaptation at 45°C (Kumar and Kumar, 2003). Similar results have been found for *S. Senftenberg*, *S. Enteritidis* and *S. Typhimurium* grown

at different temperatures (10, 20, 37, 40), with higher D-values obtained with higher growth temperature (Manas et al., 2003). However, the z-values do not seem to be affected by the growth temperature. The memory of the cell regarding heat protection persists for at least 30 minutes, as indicated by the tailing off in heat-induced gene expression, with 187 upregulated genes still remaining out of 373 upregulated genes during heat treatment at 45°C (Pin et al., 2012).

Several studies report substantial variation in the D-value of a specific strain in relation to the substrate within which it is heat-treated (Jin et al., 2008; Lang and Smith, 2008). A decrease in substrate moisture content results in increased resistance of *Salmonella* spp. to heat, mainly due to decreased heat transfer capacity (Kim et al., 2012; Bischel et al., 2016). A similar effect is found for the z-values, with materials with a dry matter content up to 30%

having a similar z-value of 5.5 ± 1 . However, when the dry matter content increases above 50%, the z-value increases to well above 30. On the other hand, the z-value has been found not to be affected by the substrate as long as a similar dry matter content is maintained (Jin et al., 2008; Murphy et al., 1999; Murphy et al., 2000) (Figure 5).

Figure 5. Increase in temperature (°C) necessary to change the decimal reduction value (D) by a factor of 10 (z-value) of 16 different *Salmonella* strains, present either individually or as a cocktail of four to eight strains, in material with different dry matter content (Jin et al., 2008; Rakowski, 2012; Soldierer and Strauch, 1991; Eckner, 1992; OrtaRamirez et al., 1997; Murphy et al., 1999; Mazzotta, 2000; Smith et al., 2001; Manas et al., 2003; Ma et al., 2009; Kim et al., 2012).

Heat-damaged salmonellae cells show great variation in the lag phase before rapid re-growth, with a lag phase of up to 20 h and with notable variation in recovery in relation to the broth used (Stephens et al., 1997). In that study, there were no correlating factors for the effect of the different broths used and duration of the lag phase. The growth temperature also affected the lag phase, e.g. 25°C resulted in a longer lag phase than 37°C (Juneja and Marks, 2006). Moreover, studies have shown that recovery of heat-damaged cells is higher under reduced oxygen conditions (George et al., 1998).

Humpheson et al. (1998) suggest that biphasic thermal inactivation kinetics should be used for heat inactivation of *Salmonella* at temperatures above 51°C. They attribute the tailing in inactivation mainly to formation of heat shock proteins that preserve the cells of a small subpopulation with a considerably higher D-value. During heat treatment, there are several possible explanations for prolonged survival of individual *Salmonella* cells, such as protective particles and uneven heat distribution in the material

(Haug, 1993). It is therefore important to know the properties of the material to be treated before planning a specific treatment method.

In summary, there is a rapid decrease in the D-value of *Salmonella* spp. with increased temperature in the range 50-60°C. The most important factor is the dry matter content of the material, as the D-value and the z-value increase with dry matter content above 30%. At the lower range of dry matter content, the most important factor for proper inactivation is achieving uniform heat distribution in all the material. For full inactivation of *Salmonella* spp., the temperature should at least be above 47.5°C and preferably above 55°C. Even at 55°C, the time required for a decimal reduction (D-value) can be above 1 hour. For validation of the process, a cocktail of several *Salmonella* spp. types should be used and the D-value and z-value for inactivation in the specific substrate should be determined. A heat map of the process is also required, to ensure that the time and temperature requirements are met within the process.

Appendix A. Methods for detection of Salmonellae

A.1 Culture methods

Pre-enrichment uses a nutritious, nonselective medium to recover sub-lethally injured salmonellae cells. Buffered peptone water (BPW) is a commonly used primary enrichment medium for environmental samples as well as in food microbiology whereas CEN/TR 15215:2006 for characterization of sludges suggest BPW supplemented with the antibiotic Novobiocin. For detection of *S. Typhi* and *S. Paratyphi* serovars, selenite cysteine (SC) broth or universal pre-enrichment (UP) broth is recommended by standards/guidelines for environmental samples as the first enrichment step (See Table 4).

Primary pre-enrichment is followed by inoculation into selective media containing inhibitory reagents to allow for further growth of salmonellae, while suppressing other bacteria. Traditionally, three types of selective enrichments are used: tetrathionate media (TT and MKTTn broth), selenite-based media (SC broth) and Rappaport media (RV, RVS and MSRV). Rappaport-Vassiliadis media (with malachite green and magnesium chloride as suppressive agent) have for the enrichment step shown more sensitive than SC and TT broth (Mooijman, 2012) and is the primary choice by several reference methods for environmental samples. However, for *S. Typhi* detection in fruit, enrichment in SC and TT broths proved much more efficient than RV medium (Hammack et al., 2008) and is also recommended by ISO 6579 for the detection of *S. Typhi* and *S. Paratyphi*. MKTTn can also recover some strains of *S. Paratyphi*, but not *S. Paratyphi* C (ISO 19250:2010). Selenite enrichment has otherwise been used most frequently for the isolation of salmonellae when direct enrichment of the sample in selective media is recommended, e.g., with stool or organ samples (Mooijman, 2012). To account for the varying selectivity of media, food microbiology reference methods have often prescribed the use of two enrichment media of different groups in parallel. Motility enrichment in semi-solid agar (e.g., MSRV; DIASALM; SMS-agar), based on swarming of motile salmonellae, functions by both enriching and indicating presumptive salmonellae in one step. Semi-solid media have shown significantly higher sensitivity compared to liquid media (De Busser et al., 2013) and MSRV is currently part of several reference methods for environmental samples.

To isolate presumptive positive salmonellae colonies, which requires inhibition of the growth of other bacteria, the selective enrichments are plated on one or more selective media in parallel. Selective plating agars rely on different selective agents, indicator systems and differential characteristics to distinguish *Salmonella* spp. from related enteric bacteria and may also to some extent be used to differentiate between different salmonellae. It is often recommended to use at least two plating media based on different selective agent and indicator systems, since no plating agar can detect all salmonellae serovars.

Chromogenic media, which exploit substrates that release colored dyes upon enzymatic hydrolysis, generally have higher specificity than traditional selective media and may be suitable for environmental samples with varying background flora. Chromogenic media helps also reduce the potential for false-positives selected for further testing. Many of the chromogenic media currently in use for salmonellae are based on C8-esterase activity or α -galactosidase, whereas β -galactosidase is used for differentiating between other enterobacteriaceae (Perry, 2017). Chromogenic media can also be sprayed onto non-chromogenic agar for further differentiation, e.g., between *Salmonella* spp. and *Citrobacter* spp. (CEN/TR 15210:2006). Fluorogenic salmonellae media generally differentiate salmonella from *E. coli* by fluorescence in the latter. Currently, there are several chromogenic agars that are sold under different brand names for which composition is not fully reported (Table 4). Several reference methods for food and environmental samples (Table 5) include Brilliant green (BG) agar, Xylose-lysine-deoxycholate (XLD) agar, and Rambach agar (Table 4) as solid media for *Salmonella* spp. isolation. XLD agar may work well for most salmonellae isolates, although H₂S-negative and/or lactose fermenting strains may result in non-typical appearance. Some salmonellae strains, including *S. Typhi* and *S. Paratyphi*, and some strains of *S. Typhimurium* and *S. Dublin*, may, however, be inhibited by brilliant green, the dye used in BG agar (Curtis and Clarke, 1994). *Salmonella Typhi*, though, will display typical growth on Miller-Mallinson (MM) medium, which also will distinguish lactose-positive *Salmonella* spp. from coliforms by their H₂S production. MM medium is suggested by [US EPA \(2010\)](#), together with Bismuth-sulfite (BS), for analyses specifically targeting *S. Typhi* in drinking water. Also, the ab-chromogenic (ABC) medium results in typical growth of *S. Typhi* and *S. Paratyphi* A and B. On Rambach agar *S. Typhi*, *S. Paratyphi* A and B, as well as strains of *S. bongori* (V) and *S. enterica* subsp. *arizonae* (IIIa) and *diarizonae* (IIIb), may appear atypical (Kuhn et al., 1994).

Presumptive salmonellae colonies isolated on solid media are subsequently subjected to several biochemical tests and serological identification. Common biochemical tests for salmonellae are based on fermentation of selected substances (e.g., citrate, glucose, mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose) and production of enzymes (β -galactosidase; decarboxylases, urease, tryptophan deaminase, tryptophanase, gelatinase etc.). Commonly applied reference methods tests include triple sugar iron agar (TSI), lysine iron agar (LIA), urease- and indole test (Table 4). Some of these tests include several aspects for identification of salmonellae, e.g., H₂S production in combination with sugar fermentation (in TSI) or L-Lysine decarboxylation (in LDC test). Test strips, such as the Analytical Profile Index kit (API 20E), include miniaturized versions of several biochemical tests with the purpose of differentiating among bacteria in the Enterobacteriace family (Tables 4 and 5).

Selective enrichment media given by standard methods for environmental samples presently includes MSRV, RVS, RV

and MKTTn for salmonellae in general and SC broth for detection of *S. Typhi* (Table 4). In addition to selective agents, an elevated incubation temperature (approximately 42°C) of the medium will further inhibit the growth of interfering background flora but is not recommended if salmonellae are assumed present in low concentrations. For samples with relatively high amounts of background flora, the semi-solid media MSRV and DIASALM, based on salmonellae motility, have shown to be very effective for the detection of salmonellae (Voogt et al., 2001). However, it has been observed that the use of semi-solid agar as a selective enrichment seems to favor the development of *S. Enteritidis* (Svastova et al., 1984; Perales and Erkiaga, 1991; Poppe et al., 1992). As selective plating media XLD is predominantly recommended by environmental standard methods (Table 6). As different culture media may have different sensitivity for different salmonella serovars, the objectives for a particular environmental analysis may determine the choice of media or that media with different specificity shall be used in parallel. *S. Typhi* and specifically *S. Paratyphi*, non-motile salmonellae and lactose positive salmonellae that also are H₂S negative, may potentially be overlooked by the standard methods (Table 6). The relevance to determine the subspecies, serotype or features as antimicrobial resistance of *Salmonella* in environmental samples may be highly dependent of the scope of the sampling (Table 4 and 5). It's worth mentioning that since the formulation, validation, review and agreement of standards typically requires several years, standard methods may not cover the latest method developments and optimal media and methodology in current research may show a significant departure from media and methods given by standards.

In the VBNC state, bacteria fail to grow on conventional growth media, but still maintain metabolic functions and cell integrity. Culture methods thus risk underestimation of total viable cells (Li et al., 2014). In the food industry, the VBNC state has been shown to be induced by disinfectants used in food hygiene (Gruzdev et al., 2011; Highmore et al., 2018) or sterilization techniques (Liao et al., 2018) and food preservation measures, e.g. dehydration, heat treatment, cold storage and acid treatment (Gruzdev et al., 2011; Gruzdev et al., 2012; Morishige et al., 2017; Xu et al., 2008). Also the environment will subject *Salmonella* spp. to stresses as osmotic-, nutritional-, heat- and cold stress. Fu et al. (2015) found that the VBNC state of *Salmonella* spp. and *Shigella* spp. in sewage sludge was induced to a higher degree (four orders of magnitude) during thermophilic anaerobic digestion compared to mesophilic digestion. As a result, the *S. Typhimurium* concentration in thermophilic digested sludge after cake storage were two orders of magnitude higher than for mesophilic digested sludge. When simulating starvation in aquatic environment, VBNC states for most of the *Salmonella* population studied was induced in 30 minutes to 5 weeks (Chmielewski and Frank, 1995; Baleux et al., 1998). Idil et al. (2011) studied the effect of UV-A and different wavelengths of visible lights on *S. Typhimurium* in seawater microcosms found that visible red light induced the largest shift to VBNC. These studies indicate that VBNC states are likely to be induced in environment, even though not frequently studied. In

general, VBNC cells have higher physical and chemical resistance than culturable cells (Li et al., 2014), which make them not just undetectable, but also increases their persistence in the environment. An extreme case is the resuscitation of *S. Typhimurium* incubated in seawater and soil microcosms for 20 years (Dhiab et al., 2010). To account for VBNC subpopulations, alternative methods for determining viability have been developed based on demonstration of cellular integrity, physiological responsiveness/metabolic activity or presence of nuclei acids (Keer and Birch, 2003). Direct count of bacterial cells with the viability indicated by staining in relation to different integrity/activity aspects is used to assess and enumerate VBNC bacterial subpopulations. Alternatives to cell counting include the use of flow cytometry and fluorescent staining techniques, the exploitation of physiological responsiveness or metabolic activity.

A.2 Immunology and nucleic acid based tests

The immunology-based methods for salmonellae detection utilize antibodies, which complex with antigens that are normally located on the cellular membrane surface of salmonellae. Depending on the formulation, the applied antibodies can serve different purposes: identification/differentiation (e.g., agglutination; serotyping; immunoprecipitation), separation/concentration (immunomagnetic separation), or detection with indirect quantification of the targeted pathogen (LFIA, EIA/ELISA). In combination with culture media, as in immunodiffusion assays, antibodies are also used as an integrated identification/confirmation step, thus reducing the time to confirm salmonellae. Agglutination and serotyping are commonly used as confirmatory steps for culture-based methods and yields clear and rapid typing down to serogroup. Lateral flow immunoassays (LFIA), often formulated as paper-based platforms, may display results within 5–30 min. Such assays have important clinical value of being rapid and easy to interpret. The small sample that is absorbed into the LFIA means that the detection limit is rather high, and that the media only can perform direct testing on water samples. The specificity is high (to serovar level), whereas sample preparation is necessary for fecal material as well as environmental samples due to the low detection limits, often $\geq 10^6$ cells per ml.

EIA/ELISA-based methods are considered to be particularly promising for rapid detection because they combine the specificity of the antibodies with the sensitivity of the enzymatic assays by coupling easily assayed enzymes to antibodies or antigens. Usually, EIA/ELISA are more selective and sensitive than agglutination or immunoprecipitation assays, but they need more time to obtain quantitative results. Still, the time required is typically less than that of most nucleic acid-based methods.

Nucleic acid-based assays detect a specific DNA or RNA target sequence within the *Salmonella* genome by matching complementary sequences of single-stranded DNA or RNA to pair with each other. Two major categories of nucleic acid-based assays are direct hybridization (DNA probe) and amplification methods, such as polymerase chain reaction

(PCR). Many primer sets have been developed to target various genes specific to both the genus and species level of *Salmonella* using both PCR and quantitative PCR (qPCR) (Table A1). These assays can target many different levels of

specificity in *Salmonella*. Often the *invA* gene is targeted for detection of *salmonella* at the genus level. Detection of species and subspecies varies widely.

Table A1. Molecular-based assays used for *Salmonella* genus detection and identification

Targeted Species	Targeted Subspecies	Target Gene	Primer Set (5'-3')	Reference
All	All	<i>invA</i>	PCR-F:GTGAAATTATCGCCACGTTTCGGGCAA, R:TCATCGCACCGTCAAAGGAACC	Rahn et al., 1992
All	All	<i>invA</i>	qPCR-F: GCGTTCTGAACCTTTGGTAATAA R: CGTTCGGGCAATTCGTTA, Probe: FAM-TGGCGGTGGGTTTTGTTGTCTTCT-TA MRA	Daum et al., 2002
All	All	<i>ttrRSBCA</i>	<i>ttr</i> -6 F: CTCACCAGGAGATTACAACATGG <i>ttr</i> -4 R: AGCTCAGACCAAAAGTGACCATC Target probe (<i>ttr</i> -5) FAM-CACCGACGGCGAGACCGACTTT- Dark Quencher	Malorney et al., 2004
<i>enterica</i>	All	<i>iroB</i> -192	F: CCAAGAGATCTGGCGTGGATA R: TATTGCATGGAGATAACCGGCT	Murphy et al., 2007
<i>enterica</i>	All	<i>iroB</i> -12	F: GGRACAAAAATGGGGMACTTCT R: AAGGGGAGGRTAGACGATGA	Barbau-Piednoir et al., 2013
<i>enterica</i>	Enterica	<i>safC</i> -10	F: CACTAAAAAGTCGTCTGACTCTGG R: CGGTAAATGGCACACTATCAAAT	Barbau-Piednoir et al., 2013
<i>enterica</i>	Enterica	<i>safC</i> -13	F: ATGATGACAGCTACGGACTGAGTT R: CTGTTTTGATTCCAGTTCAATGAC	Barbau-Piednoir et al., 2013
<i>enterica</i>	Enterica	STM0296	F: AMGCCCATCCCTTCAATACAG R: GTGATYACGCCGAGCATAACC	Barbau-Piednoir et al., 2013
<i>enterica</i>	Typhi	STY0201, <i>staG</i>	F: CGCGAAGTCAGAGTCGACATAG R: AAGACCTCAACGCCGATCAC Probe: FAM-CATTTGTTCTGGAGCAGGCTGACGG-TAMRA	Nga et al., 2010
<i>enterica</i>	Paratyphi	SSPA2308	F: ACGATGATGACTGATTTATCGAAC, R: TGAAAAGATATCTCTCAGAGCTGG , Probe:CCATACAATTTCACTTATTGAGAATGCGC-BHQ5[	Nga et al., 2010
<i>enterica</i>	Paratyphi	SPC0869	F: CTGGCTGACACATGAACAAATC, R: CCTGAGAACGAGTCAGGTTTAC, Probe: CY5-TGTACGACTGCAAACGCCAAAGTC- BHQ2	Nair et al., 2019

Direct hybridization assay use a labeled DNA probe complementary to the target sequence of a DNA or RNA molecule present in the target bacteria and quantify the DNA by correlation of signals measured from labels, labeled substrates, or sub-products (Lee et al., 2015; Mozola et al., 2013). The analytical detection technique used depends on the characteristics of the label (enzymatic, radioisotope, fluorescence, etc.), but colorimetric assays are the most common.

Laboratory automation and miniaturized tests may be important future complementary technologies, especially for rapid screening or identification purposes. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) allows ionization of biomolecules as DNA, proteins, peptides and sugars, compounds, which tend to be too fragile to be ionized by conventional MS. Used for bacterial identification, it mainly analyzes the composition of proteins of low molecular weight in cell lysate or whole cells, which means that, e.g., biomarker pre-fractionation, digestion, separation, or cleanup is not required. Main benefits of MALDI-TOF MS include reduced time for confirmation (1 hour) and reduced cost of identification and screening of multiple colonies at the same time. However, since it is still dependent on culture methods for isolation, it mainly serves as an identification tool. Studies applying MALDI-TOF-MS for

salmonellae indicate that the method provides high accuracy in identification of salmonellae at species level, but currently is limited in capacity to type or subtype salmonellae serovars (Kang et al., 2017). It is suggested that MALDI-TOF MS can be used in conjunction with culture media and to some extent compensate for a lack of specificity of some culture media for salmonellae giving an overall cost effective and rapid test procedure when capital investments are made (Perry, 2017). Biosensors are analytical devices aiming to perform chemical or biological analysis theoretically with no considerable sample preprocessing and comprises a bio-receptor integrated with a signal transducer (Silva et al., 2018). Bio-receptors for pathogen detection recognize and react with enzymes, whole cells, specific proteins, antibodies or their fragments, nucleic acids or related substances and since read by a transduced signal easy to interpret. However, even though development is ongoing, the examples of commercial biosensors currently available in the market seem to be limited.

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